Novel strategies for making myasthenia less gravis : targeting plasma cells and the neuromuscular junction

MAASTRICHT UNIVERSITY

Novel strategies for making myasthenia less gravis

Targeting plasma cells and the neuromuscular junction

Alejandro Martin Gomez

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“It always seems impossible until it’s done”

N. Mandela

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Abbreviations

2D-DIGE	Two-dimensional difference in-gel electrophoresis
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AMR	Antibody-mediated transplant rejection
Btz or Bz	Bortezomib
Bw	Body weight
C5	Complement factor 5
CAIII	Carbonic anhydrase III
CFA	Complete Freund's adjuvant
CMAP	Compound muscle action potential
CMS	Congenital myasthenic syndromes
Dok-7	Downstream of kinase 7
EAMG	Experimental autoimmune myasthenia gravis
EM	Electron microscopy
EMG	Electromyography
EOMG	Early-onset myasthenia gravis
EPP	Endplate potential
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FSC	Forward scatter
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HRP	Horseradish peroxidase
IF	Immunofluorescence
iFBS	Heat-inactivated fetal bovine serum
IVIg	Intravenous immunoglobulin
Lrp4	Low-density lipoprotein receptor-related protein 4
mAb	Monoclonal antibody
MAC	Membrane attack complex
MEPP	Miniature endplate potential
MG	Myasthenia gravis
MGFA	Myasthenia gravis foundation of America
MIR	Main immunogenic region
MM	Multiple myeloma
MMF	Mycophenolate mofetil
MuSK	Muscle specific kinase
NMJ	Neuromuscular junction
PBMC	Peripheral blood mononuclear cell
PEx	Plasma exchange
PI	Propidium iodide

PN	Peripheral neuropathy
PTB	Phosphotyrosine binding
PWM	Pokeweed mitogen
Rapsyn	Receptor-associated protein of the synapse
RIA	Radioimmunoassay
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SSC	Side scatter
SV2	Synaptic vesicle protein 2
tAChR	*Torpedo* acetylcholine receptor
Tid1	Tumorous imaginal disk 1
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TTP	Thrombotic thrombocytopenic purpura
VGSC	Voltage-gated sodium channels

General Introduction

Alejandro M. Gomez

Myasthenia gravis, an archetypal autoimmune disease

The complex adaptive immune system of vertebrates has evolved to specifically recognize foreign antigens (mainly proteins from microbes and parasites) and eliminate them upon infection. The effector cells responsible for this task, B- and T-lymphocytes, have the potential of recognizing virtually any antigen by expressing randomly-mutated receptors at their surface, a feature that also poses risk for self-antigen recognition [1]. Consequently, the immune system developed fine selection processes to eliminate or inactivate lymphocytes that could react against host proteins; in other words, the adaptive immune system only allows in the circulation lymphocytes that "tolerates" the host antigens [2]. However, as with most of biological processes, these tolerance mechanisms can fail and, in such cases, self-reactive immune responses are mounted against "self"-tissues, which is the basis for the development of autoimmune diseases [3].

Myasthenia gravis (MG) is one of the best characterized autoimmune diseases. Patients affected by MG mount an immune response against the postsynaptic component of the neuromuscular junction (NMJ), which is the synapse between a motor nerve terminal and the muscle; thereby blocking neuromuscular transmission and, thus, impairing muscle contraction. In this regard, the clinical presentation of MG is well explained by its own name: "myasthenia" means muscle weakness (one of the main symptoms, in addition to fatigability), and "gravis" means severe (because in some cases the weakness can be incapacitating or even lethal). One important aspect of MG is that the targets for the autoimmune response are well defined, with responses against the muscle acetylcholine receptor (AChR, 85%), or muscle specific kinase (MuSK, 10%), or low density lipoprotein receptor-related protein 4 (Lrp4, about 1-2%), which also facilitates its clinical diagnosis [4]; the rest of patients (~4%) have no detectable antibodies and are termed "seronegative" or idiopathic MG-patients. Moreover, several lines of evidence have demonstrated that these antibodies, particularly anti-AChR antibodies, are the sole mediators of the clinical symptoms. They bind and activate complement at the NMJ [5], they accelerate AChR degradation [6], they can passively transfer the disease to animals [7] or to neonates (neonatal MG) [8], and their removal by plasmapheresis and

immunosuppression ameliorates muscle weakness [9]. This is usually not the case for many of the autoimmune diseases so far recognized, which tend to react against several auto-antigens and, in many of them, it is also difficult to clearly individualize the causal relationship between autoimmune response and pathology.

For the abovementioned reasons, MG has been traditionally regarded as a one of the prototypical antibody-mediated autoimmune diseases [10]. In addition, its target organ (the NMJ) is relatively accessible and easy to manipulate for both research in MG patients and animal models, which helped to boost research in the pathophysiology of MG already in the late 70's. This was also fostered by its early recognition as an autoimmune condition in the 60's, when the concept of autoimmune diseases was just emerging [11].

Historical overview of myasthenia gravis

The first reports of myasthenia gravis date back to the 17th century, with the description in England and the American colonies of a debilitating condition of the bulbar muscles that did not involve the central nervous system. One such case was the Native American chief Openchancanough, from whom it was accounted by early colonists that "was unable to walk and his eyelids were so heavy that he could not see unless they were lifted up by his attendants" [12]. More comprehensively documented is the report of the Oxford-born physician Thomas Willis who, in 1672, described the case of a woman with recurrent weakness in her bulbar muscles that prevented her from speaking after prolonged use of her voice; he referred to this condition as some kind of "spurious palsy" [13].

By the end of the 19th century several cases of the spurious palsy were documented, which were usually regarded as atypical presentations of bulbar paralysis due to the lack of neuronal loss. However, in 1893, MG gained recognition as a condition distinct from bulbar paralysis when the Polish physician Samuel Goldflam provided a complete account of the symptoms, after carefully reviewing the cases described until then [14]. The name of this novel muscle disease emerged a few years later when Friederich Jolly, reporting two cases in a conference in Berlin, referred to his patients as suffering from "myasthenia gravis pseudo-paralytica" [15]. In the upcoming years, the relation between MG and the impairment of neuromuscular transmission, instead of the

central nervous system, would become more evident. Initial observations indicated that repetitive nerve stimulation could trigger muscle weakness in MG and later, in the 1930's, Dr. Mary Walker successfully treated MG patients in London's St. Alfege's Hospital by enhancing neuromuscular transmission with acetylcholinesterase inhibitors [16]. The story goes that she attempted this treatment after noticing that the clinical picture of MG patients resembled that of curare poisoning, which was usually treated with cholinesterase inhibitors [17].

In the early 20th century, the immunological nature of MG started to be slowly unveiled. Despite the fact that early physicians, such as Willis, tried to explain its possible causes by referring to fluctuations of a circulating substance called "explosive copula", which could be associated with immune components, one of the first empiric observations pointing at the immune system was made by Edward Buzzard; he reported an accumulation of lymphocytes in both the affected muscles and the thymus of MG patients [18]. Subsequently, several papers exposed that MG patients frequently presented thymic alterations [19-22], driving attention to the thymus as a key organ in the pathogenesis of MG. Shortly thereafter, the first thymectomy was successfully performed in Switzerland in 1911 and, by 1944, the heart surgeon Alfred Blalock had thymectomized 20 MG patients at the Johns Hopkins hospital in Baltimore, reporting his findings in an original paper [23]. Since the 1960's, thymectomy has been one of the standard treatments for MG, along with cholinesterase inhibitors.

It was not until the 1950-1960's that the first theories of an autoimmune component in the pathology of MG were formulated. John Simpson, a Scottish physician, is credited for publishing the seminal paper arguing that MG was caused by an autoimmune reaction to the motor endplates, after studying the transient effects of neonatal myasthenia in children of MG mothers and the association of MG with other autoimmune diseases [24]. This hypothesis received fundamental support about a decade later when Patrick and Lindstrom, in an attempt to generate antibodies against purified acetylcholine receptors (AChRs) by injecting the purified protein into rabbits, serendipitously created the first animal model for MG (experimental autoimmune myasthenia gravis, EAMG) [25]. This model was also crucial to define that MG was

mediated by the autoantibodies, since the disease could be transferred by serum to naive animals [26]. A few years later, the development of a radioimmunoassay in which radioactive-labeled AChRs were immunoprecipitated by anti-AChR antibodies, allowed for measurement of autoantibody titers in serum of MG patients and helped to confirm that anti-AChR antibodies were indeed pathogenic [27].

In the last decades of the 20th century, immunological research in MG unfolded. The contribution of different immunoglobulin subclasses to the pathological changes was well-characterized and complement-mediated damage at the neuromuscular junction was more clearly defined as one of the hallmarks in MG [28]. Also, increased internalization of cross-linked AChRs by bivalent antibodies [29] and blockade of acetylcholine-binding sites [30] were regarded as important pathological mechanisms of the disease. Most importantly, the increased knowledge of the pathology behind MG helped to diversify the treatment available for patients. In particular, immunosuppressive therapy, which started already in the 1960's with the use of corticosteroids and azathioprine, consolidated as the standard treatment for MG, while research on new immunosuppressive drugs started to flourish [31]. In the 2000's, the discovery of new autoimmune targets in seronegative patients, muscle specific kinase (MuSK) [32] and low density lipoprotein receptor-related protein 4 (Lrp4) [33], revealed that MG in fact encompassed distinct autoimmune conditions and paved the way for a more specific treatment of patients.

Treatment of myasthenia gravis

The incidence (30 cases/1,000,000/year; all ages, worldwide) [34] and prevalence (20/100,000, in USA, 2003 [35]; and 10/100,000 in the Netherlands, 2007 [36]) of MG are relatively low, but it still represents a considerable number of patients in the general population (about 60,000 patients in USA; and 1,500 patients in the Netherlands) [35, 36]. As with most of autoimmune diseases, the risk for development of MG is higher in females below 40 years of age (early-onset MG) and is almost equal for both sexes after 40 years (late-onset MG) [37]. Taking into consideration that MG is a chronic and potentially disabling condition, adequate and efficient treatment strategies are important for both improving the quality of life of the patients and reducing the economic burden of

treatment.

For the most part, MG patients are managed by a neurologist, who will perform the electrophysiological tests to detect alterations in neuromuscular transmission that are typical of MG, and later confirm the diagnosis by testing for autoantibodies. In most cases, one of the first clinical signs of MG is ptosis, which is the drooping of one or both eyelids due to ocular muscle weakness. In some cases, symptoms will be confined to the ocular muscles (ocular MG) but, in the majority of patients, muscle weakness will eventually spread to other muscles as well; such as the limbs, neck, bulbar and respiratory muscles (generalized MG). When respiratory muscles are involved, MG poses a potentially life-threatening risk to the patient, since severe weakness in such muscles usually requires hospitalization and assisted ventilation, a so-called “myasthenic crisis”, which also increases the risk of respiratory track infections. According to muscle involvement and severity, MG patients are classified following the recommendations of the Myasthenia Gravis Foundation of America (MGFA) in five different classes (Table 1) [38], what also helps to define which treatment is the more adequate to follow.

One of the first-line and most frequently used treatments for MG patients is thymectomy, the removal of the thymus; though it is only indicated in patients <60 years old, or older patients with an associated thymoma [31]. Thymectomy usually improves the clinical outcome of MG patients after immunosuppressive treatment, particularly in newly-diagnosed and severely affected patients; although a randomized controlled clinical trial to assess the real benefits of thymectomy in MG is still missing [39]. Moreover, the precise mechanisms by which thymectomy ameliorates MG remain largely unknown, even though the links between thymus and MG stretch back to almost 100 years ago. Possible explanations are an impaired central tolerance for T-cells that is ameliorated by thymectomy [40, 41] and, since both epithelial thymic cells and myoid thymic cells express AChR, the elimination of a potential site for constant activation of autoreactive B- and T-cells [42].

Table 1. MGFA Clinical Classification

Class I	Any ocular muscle weakness May have weakness of eye closure All other muscle strength is normal
Class II	Mild weakness affecting other than ocular muscles May also have ocular muscle weakness of any severity
IIa	Predominantly affecting limb, axial muscles, or both May also have lesser involvement of oropharyngeal muscles
IIb	Predominantly affecting oropharyngeal, respiratory muscles, or both May also have lesser or equal involvement of limb, axial muscles, or both
Class III	Moderate weakness affecting other than ocular muscles May also have ocular muscle weakness of any severity
IIIa	Predominantly affecting limb, axial muscles, or both May also have lesser involvement of oropharyngeal muscles
IIIb	Predominantly affecting oropharyngeal, respiratory muscles, or both May also have lesser or equal involvement of limb, axial muscles, or both
Class IV	Severe weakness affecting other than ocular muscles May also have ocular muscle weakness of any severity
IVa	Predominantly affecting limb and/or axial muscles May also have lesser involvement of oropharyngeal muscles
IVb	Predominantly affecting oropharyngeal, respiratory muscles, or both May also have lesser or equal involvement of limb, axial muscles, or both
Class V	Defined by intubation, with or without mechanical ventilation, except when employed during routine postoperative management. The use of a feeding tube without intubation places the patient in class IVb.

** Adapted from [38]*

Newly-diagnosed AChR-MG patients will usually receive symptomatic treatment in the form of pyridostigmine (Mestinon), regardless of their MGFA classification [43, 44]. This drug inhibits the activity of acetylcholinesterase (AChE) at the synaptic cleft and, therefore, delays the breakdown of acetylcholine and prolongs the stimulation of AChRs at the muscle membrane. In most cases, however, AChE-inhibitors treatment will be combined with certain form of immunosuppression, from which several options exist according to clinical severity and responsiveness to previous treatments. The most widely

used immunosuppressive drugs in MG are corticosteroids, prednisone/prednisolone in particular, because they improve the clinical condition in about 70% of patients (observational studies, no double-blinded placebo controlled trial) albeit usually after 3-6 months after initiation of treatment, and they are orally-administered and relatively inexpensive [43, 45]. However, corticosteroid treatment carries complications and side effects: first, severely affected patients will initially receive high doses of prednisone, which can worsen the muscle weakness and, consequently, hospitalization is required during the first days of treatment; second, long-term treatment with prednisone can lead to severe adverse effects such as osteoporosis (particularly in the elderly), weight gain, glucose intolerance, hypertension and gastrointestinal complications [46]; and third, patients might be or become unresponsive to corticosteroids treatment [47]. In order to reduce the dose of corticosteroids, physicians usually combine prednisone with a second immunosuppressant that, in most cases, is azathioprine. Azathioprine is a purine analogue that inhibits the *de novo* synthesis of purines and therefore prevents the proliferation of both T- and B- lymphocytes rather specifically, because they lack a salvage pathway for purine recycling [48]. The main advantage of azathioprine treatment is that it is well-tolerated and easy to administrate, the most common adverse effects are a flu-like syndrome and transient hepatotoxicity, though in rare cases bone marrow suppression has been associated with long-term treatment. The main disadvantage of azathioprine is its long induction period for improvement of the clinical status, with initial responses starting at about 6 months and peaking at 12-24 months [46, 49]. Still, the combination prednisone/azathioprine is the most commonly used immunosuppressive treatment for MG patients, and it is likely to remain this way in the upcoming years.

Alternatively, different “second line” immunosuppressants can be combined with prednisone instead of azathioprine, or used instead of prednisone for corticosteroid-unresponsive patients. One of such is mycophenolate mofetil (MMF), which, like azathioprine, inhibits the proliferation of T- and B- lymphocytes by blocking purine synthesis, though it does so more specifically than azathioprine, and targeting activated lymphocytes in particular [50]. MMF is still frequently used in MG patients because of its mild adverse-effect profile and good tolerability [49], despite not having shown beneficial

effects on randomized-controlled trials [51, 52]; probably because these trials had drawbacks in their design (such as duration of the study and pre-treatment history of the patients) that undermined the prednisone-sparing effects of MMF [53]. Other second line treatments include: cyclosporine A and tacrolimus, both calcineurin inhibitors that block cytokine signaling (specially IL-2) in activated T-cells [54, 55]; cyclosphosphamide, a nitrogen mustard alkylating agent that induces cell death and prevents cell replication by DNA modification; and methotrexate, a folate analog that inhibits the metabolism of folic acid and impairs the synthesis of thymidine and purines, thereby blocking cell replication, in addition to exerting anti-inflammatory effects [56]. The majority of these drugs have been proven beneficial in MG when combined with corticosteroids, though their adverse-effects tend to be more frequent and severe than with azathioprine and MMF (particularly for cyclosporine A and cyclophosphamide) [46] and, therefore, their use is reserved only for steroid-resistant patients.

In the event of a myasthenic crisis, one of the therapeutic goals is to rapidly clear the serum from the pathogenic autoantibodies and, therefore, the preferred treatment of choice is either plasma exchange (PEx) or intravenous immunoglobulin (IVIg). PEx leads to a transient fall of anti-AChR antibody titers that translates into a rapid improvement of clinical symptoms, but this effect is short-lived and autoantibody titers will rise again about 2 weeks after a course of PEx [57]. In general, the procedure is well tolerated and the side effects are mild (chills, nausea, and vomiting), though complications such as pulmonary embolism, hypotension and sepsis may occur. Moreover, given its requirement for sophisticated apparatuses and technical expertise, it is an expensive procedure and is normally unavailable in small community hospitals [58]. Alternatively, IVIg can be used for the management of myasthenic crisis, or in refractory patients, since it has been demonstrated that its efficacy for improving clinical symptoms is similar to that of PEx [59]; additionally, side effects are considered to be fewer and less severe with IVIg [60]. The immunomodulatory mechanisms that might contribute to the improvement of clinical symptoms with IVIg are various and include: Fc receptor blockade, anti-idiotypic antibodies, modulation of cytokine production, and inhibition of complement deposition on target tissues [61]. Still, as with PEx, the benefits from IVIg therapy are transient and,

therefore, repeated infusions are needed to avoid relapses [62, 63]. In addition, antigen-specific plasma apheresis of anti-AChR antibodies is another interesting treatment alternative for severely-affected MG patients, though it is still experimental [64].

In the past decades, a large number of new immunosuppressive agents have been tested or are currently under investigation as potential new therapies for MG. Examples of these agents are: etanercept, a recombinant tumor necrosis factor-α (TNF- α) receptor blocker [65]; terbutaline, a β2 agonist [66]; eculizumab, a monoclonal antibody against the complement factor 5 (C5) [67]; and the C5 inhibitor rEV576 [68]. The majority of them showed significant improvement of muscle strength in MG, though with varying degrees, in small clinical trials or animal models. One of these new agents, however, stands out from the crowd in terms of efficacy and experience in the clinics: rituximab. This chimeric mouse-human monoclonal antibody directed against CD20 eliminates circulating B-cells and has proven to be efficacious in the treatment of MuSK-MG. B-cell depletion with rituximab was originally intended for the treatment of B-cell malignancies but in the last years its use expanded also to autoimmune diseases [69]. The main advantages of rituximab treatment are its quick onset of action (as early as 2 weeks) [70] and relatively good safety profile. Rituximab's main adverse effect is infusion-related reactions, though severe neurological complications (e.g. multifocal leukoencephalopathy) and systemic infections have been also described in lupus and rheumatoid arthritis patients [69]. However, despite the initial enthusiasm for using rituximab to treat refractory MG, it has been recently reported that its long-term efficacy for refractory AChR-MG is not outstanding; since patients improve their clinical condition only moderately and anti-AChR autoantibody titers are not very much reduced by rituximab [70]. Nevertheless, rituximab reduces autoantibody titers and significantly improve clinical symptoms in MuSK-MG, making it a promising treatment alternative for this subgroup of patients [70].

The problem of treatment-refractory myasthenia gravis

The prognosis of MG has improved remarkably since the initial immunosuppressive treatments in the 1960's and, currently, it is more or less accepted that myasthenia gravis

is not as “gravis” as it was in the pre-immunosuppressant drugs era. In addition, the widespread use of thymectomy for EOMG patients has increased the rate of patients with clinical improvement and lower doses of corticosteroids as maintenance therapy, and even increased the rate of patients with complete stable remission (absence of symptoms without immunosuppressive treatment) [71, 72]. However, despite the wide array of efficacious treatment options available, some MG patients remain unresponsive to immunosuppression and are in need of novel therapeutic alternatives. These “refractory” MG patients usually undergo chronic palliative treatments, such as PEx or IVIg, which are scheduled according to disease severity and grade of response to other treatments; or they are treated with more experimental drugs with severe side effects. As a consequence, refractory patients are at a higher risk for experiencing severe complications or myasthenic crises and, in such a case, effective treatment alternatives are virtually nil, which increases the chances of fatal complications.

As mentioned, the number of novel immunosuppressive/immunomodulatory agents has expanded and has increased the options for treating refractory MG patients. Nevertheless, most of these novel immunosuppressive agents are not primarily intended for the reduction of the pathological anti-AChR autoantibodies and, those who do directly target the humoral immune system, such as rituximab, have not shown effective autoantibody reduction in AChR-MG. In this regard, the rather poor of effects of rituximab in antibody titers can be possibly explained by the survival of long-lived plasma cells to this treatment: these cells lack CD20 on their surface, can live up to several years at survival niches in the bone marrow, are the main producers of antibodies, and resist most of the currently available treatments for MG. Long-lived plasma cells are, thus, an attractive candidate to explain the resistance of some MG patients to treatment and, consequently, they are also a promising therapeutic target. Additionally, elimination of autoreactive plasma cells in patients with high autoantibody titers might be an effective approach to rapidly reduce titers until standard immunosuppression with corticosteroids and azathioprine takes effect.

Targeting the immune system and the target organ

Diseases, and by extension autoimmune diseases, result from the interaction of an infective agent (e.g. pathogen) and the body's effector agent (i.e. immune system), in the context of a specific target organ, tissue, or cell. The effects of both the pathogen trying to replicate and the immune system attempting to eliminate it will influence the homeostasis of the target organ, inducing phenotypic changes that are the signs of a disease. In evolutionary terms, there are roughly two main strategies for avoiding disease once a host has been infected. One strategy is the "resistance" to an infection, which refers to the capacity of the immune system to eliminate or prevent the infection. The second strategy is the "tolerance" of the host to the infection, which reduces the negative impact of the pathogen (or the immune response to eliminate the pathogen) in the overall fitness of the target organ [73].

The idea of disease tolerance in animal immunology has emerged primarily in the past decade and it has not been comprehensively addressed by the scientific community. Research on disease tolerance mechanisms has potential applications to both increase our knowledge about pathophysiology of diseases and, more importantly, to develop new tools for novel therapies [74]. For instance, it has been recently demonstrated that hemoglobin mutations in *Plasmodium*-resistant individuals confer a survival advantage not much by impairing the parasite's replication in red blood cells, but mainly by inducing resistance to the toxic effects of hemolytic by-products in malaria [75]. Thus, such mutations seem to have been evolutionary selected for enhancing the host's tolerance to the infection rather than for preventing it. In this regard, manipulations of the enzymatic machinery that is activated by mutated hemoglobin can be a promising therapeutic alternative to improve symptoms in severe malaria [76, 77].

Traditionally, autoimmune diseases have been treated by a rather unspecific modulation of the immune system, in an attempt to concomitantly decrease the autoimmune response or its effector mechanisms. In other words, treatment of autoimmune conditions has been largely focused on diminishing the capacity for infection-resistance of the immune system, either as a whole or only in a certain fraction of it. Because of the success of these immunomodulatory treatments, little attention has been

paid to the response of the target organ to the autoimmune attack. As a consequence, manipulating the tolerance of the host to the autoimmune attack has, for the most part, been neglected as a therapeutic alternative in autoimmune diseases.

In order to apply concepts of disease tolerance for understanding autoimmune diseases, it is important to consider the tolerance capacity of different organs to an immune attack. Organs such as the brain and the heart have a low tolerance capacity since their ability for tissue regeneration is poor and they have a low resistance to hypoxia; consequently, they are very susceptible to inflammation-induced damage. Also, tissues with poor repair mechanisms (i.e. low cell turnover, high sensitivity to oxidative stress and slow tissue regeneration, susceptibility to fibrosis, among others) are predisposed to experience autoimmunity-related sequels [73].

When it comes to muscle tissue, the embryonic development of NMJs is well characterized, but very little is known about their maintenance and repair mechanisms during adulthood. Such knowledge might provide new tools for understanding and treating MG. For instance, it has been shown that the expression of the AChR-anchoring protein rapsyn is increased in the muscle of EAMG animals [78], and increasing its levels at the NMJ of rats reduces their susceptibility to the autoantibody attack in EAMG [79, 80]. In addition, mutations in AChR-clustering proteins of the NMJ cause of a group of conditions characterized by impaired neuromuscular transmission and muscle weakness, which are termed congenital myasthenic syndromes (CMS) [81]. This also implies that the loss of these AChR-clustering proteins in MG could aggravate even more the disease by facilitating the loss of AChRs or delaying the recovery from the autoantibody damage. It is plausible to consider, thus, that such proteins are intrinsically important for the tolerance capacity of the NMJ. Consequently, their manipulation could offer new treatment strategies that could be valuable for improving symptoms in MG patients, particularly those who are severely affected.

Aim and outline of this thesis

The six chapters presented on this thesis are aimed at investigating new strategies for a more holistic treatment of MG, focusing not only on the immune system, but also on the

target organ (the muscle). In our search for novel therapies, we explored the effects of the autoimmune attack in the muscle and evaluated the therapeutic potential of the, so far neglected, plasma cells.

Chapter 1 gives a comprehensive introduction to the normal structure, organization and physiology of the NMJ, and the pathological alterations in both MuSK- and AChR-MG. Emphasis is given to the contribution of different IgG isotypes to the pathophysiology of MG and particular interest is placed on explaining how the loss of AChR-associated proteins can contribute to disease severity. In addition, the main animal models of MG are described.

Chapter 2 characterizes the response of the muscle to an autoimmune attack in the chronic EAMG model. For this purpose, we studied the differentially expressed proteins in tibialis anterior muscles of chronic EAMG rats. The muscle's proteome was analyzed by two-dimensional difference in-gel electrophoresis (2D-DIGE) technology and the changes in relevant proteins were confirmed by Western blotting.

Chapter 3 investigates the role of the muscle protein Dok-7 in both the susceptibility to and the recovery from a passive-transfer EAMG model. Since Dok-7 is fundamental for the development of the NMJ, and its mutations are one of the major causes of CMS, we hypothesized that silencing its expression with siRNAs would increase the susceptibility to EAMG and delay the recovery of the NMJ from the autoantibody attack.

Chapter 4 elaborates on the importance of the main antibody-producing cell, the plasma cells, in the treatment of autoimmune diseases, with emphasis in the treatment of MG. It also provides an overview of the *in vitro* and *in vivo* experience with the proteasome inhibitor bortezomib in non-neoplastic conditions; one of the few compounds that can kill plasma cells and is also approved for clinical use in patients. Additionally, it discusses the rationale of bortezomib's potential applications for the treatment of MG.

Chapter 5 investigates the effects of proteasome inhibition with bortezomib in the treatment of chronic EAMG rats. It demonstrates the toxic effects of bortezomib on plasma cells *in vivo* and shows that depletion of plasma cells can significantly reduce autoantibody titers and, thus, improve the clinical condition.

Chapter 6 studies the *in vitro* effects of proteasome inhibition on cultured thymic cells from early onset MG patients. It shows that administration of bortezomib, but not corticosteroids, eliminates autoimmune human plasma cells from the cultured thymic cells, thereby blocking autoantibody and IgG production.

The main contents of these chapters are summarized and illustrated in Figure 1.

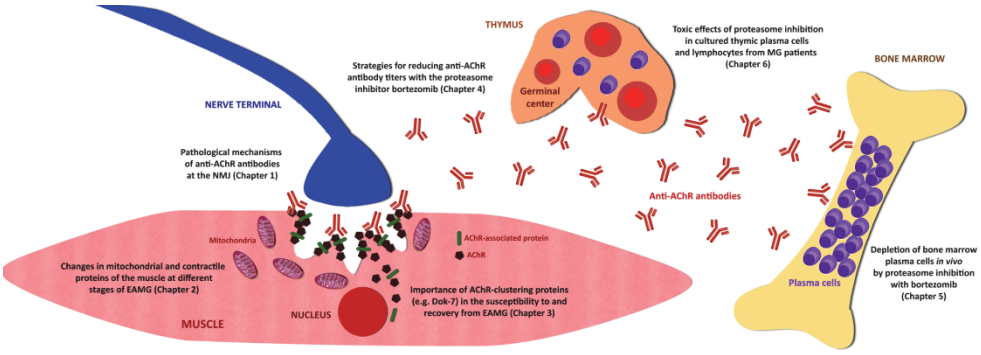

Figure 1. Illustrative overview of the different chapters presented in this thesis. This simplified representation of the features of MG most directly related to this thesis includes: the generation of germinal centers and autoimmune plasma cells in the thymus of MG patients, which were used to test the efficacy of the proteasome inhibitor bortezomib (Chapter 6); the accumulation and prolonged survival of autoimmune plasma cells in the bone marrow, which were depleted by treatment with bortezomib in the EAMG rat model (Chapter 5); high levels of anti-AChR antibodies in serum and novel strategies for reducing them (Chapter 4); pathological changes at the NMJ due to the binding of autoantibodies in MG (Chapter 1); changes in the proteomic profile of muscle fibers at different disease stages of EAMG (Chapter 2); relative importance of the AChR-clustering machinery (in particular the cytoplasmic protein Dok-7) in the susceptibility to and recovery from the autoantibody attack in EAMG (Chapter 3).

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Chapter 1

Antibody effector mechanisms in myasthenia gravis - Pathogenesis at the neuromuscular junction

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Abstract

Myasthenia gravis (MG) is an autoimmune disorder caused by autoantibodies that are either directed to the muscle nicotinic acetylcholine receptor (AChR) or to the muscle specific tyrosine kinase (MuSK). These autoantibodies define two distinct subforms of the disease - AChR-MG and MuSK-MG. Both AChR and MuSK are expressed on the postsynaptic membrane of the neuromuscular junction (NMJ), which is a highly specialized region of the muscle dedicated to receive and process signals from the motor nerve. Autoantibody binding to proteins of the postsynaptic membrane leads to impaired neuromuscular transmission and muscle weakness. Pro-inflammatory antibodies of the human IgG1 and IgG3 subclass modulate the AChR, cause complement activation and attract lymphocytes; together acting to decrease levels of the AChR and AChR-associated proteins and to reduce postsynaptic folding. In patients with anti-MuSK antibodies there is no evidence of loss of junctional folds and no apparent loss of AChR density. Anti-MuSK antibodies are predominantly of the IgG4 isotype which functionally differs from other IgG subclasses in its anti-inflammatory activity. Moreover, IgG4 undergoes a posttranslational modification termed Fab arm exchange which prevents cross-linking of antigens. These findings suggest that MuSK-MG may be different in etiological and pathological mechanisms from AChR-MG. The effector functions of IgG subclasses on synapse structure and function are discussed in this review.

Introduction

Myasthenia gravis (MG) is an autoimmune disease caused by autoantibodies against proteins in the postsynaptic membrane of the neuromuscular junction (NMJ) [1,2]. Several lines of evidence have clearly demonstrated that antibodies against the acetylcholine receptor (AChR) cause the disease in approximately 85% of MG patients [1]. Autoantibodies against the muscle specific tyrosine kinase (MuSK) have been detected in up to 70% of MG patients without autoantibodies against the AChR [2]. MuSK is essential for the development and maintenance of the NMJ [2-4] since it is required for AChR clustering. Together, AChR-MG and MuSK-MG cover more than 90% of MG cases.

The remaining patients also have an autoimmune disorder, since they, too, benefit from immunosuppression or plasmapheresis [5]. Historically, the term "seronegative MG" was used for MG without anti-AChR antibodies, but with the discovery of MuSK antibodies in many seronegative patients, this term has become confusing. Therefore, we will use the following nomenclature in this review: "AChR-MG" for myasthenia gravis with anti-AChR antibodies; "MuSK-MG" for myasthenia gravis with anti-MuSK antibodies; "idiopathic MG" for patients with (autoimmune) myasthenia gravis, but (so-far) undetectable levels of anti-AChR or anti-MuSK antibodies (Table 1.1).

In AChR-MG patients, antibodies against many other antigens can be found [6]. Their role in the pathogenesis and their contribution to the clinical symptoms of MG are not completely understood yet. Many of these antibodies are not specific for MG and they also occur in patients with other autoimmune diseases. Some autoantibodies are associated with malignancies of the thymus [7]. Thymic abnormalities such as hyperplasia and thymoma are significant risk factors for AChR-MG [8, 9].

Idiopathic MG is more similar to AChR-MG than to MuSK-MG regarding thymic pathology [10] and clinical features [11]. Since idiopathic MG patients have the same clinical presentation as patients with AChR-MG, it is possible that they produce AChR autoantibodies at a very low level or with low affinity, which makes these autoantibodies undetectable for conventional AChR antibody assays [12-14]. Alternatively, autoantibodies might be directed against other neuromuscular proteins in idiopathic MG patients.

Table 1.1. MG and related animal models

MG in humans		MG in animals	Animal models	
Chronic	Transient	Chronic	Active immunization	Passive immunization
AChR-MG	**Neonatal AChR-MG**	**AChR-MG**	**AChR-EAMG**	**Passive transfer AChR-MG**
human IgG1, human IgG3	[140-143]	cats [144], dogs [145]	rabbit [121], rats, guinea pigs [123], rhesus monkeys [124]	mice [115], rhesus monkeys [49], rats [146]
MuSK-MG	**Neonatal MuSK-MG**		**MuSK-EAMG**	**Passive transfer MuSK-MG**
human IgG4	[147, 148]		rabbits [116], mice [117]	mice [118]
Idiopathic MG "seronegative MG"				

The structure and physiology of the NMJ is complex, but well understood. The pathogenic changes at the NMJ in MG are interesting also from an immunological point of view, since it provides a clear example of autoantibody effector functions on the target tissue. Immunotherapies for MG patients do not only depend on suppressing or modulating the autoimmune processes, but also on the repair processes and plasticity of the NMJ. Therefore, we first review the normal structure and function of the NMJ and secondly discuss the changes at the NMJ as a function of IgG isotype and specificity.

Organization and physiology of the NMJ

For understanding the pathophysiology in MG, it will be useful to recall some basic facts of neuromuscular transmission. The NMJ is a chemical synapse that transmits a signal from the motor nerve to the postsynaptic region of the muscle fiber (Fig. 1.1 and 1.2). The postsynaptic membrane contains a high density of AChR (Fig. 1.3) which is a ligand-gated

ion channel [15]. The impairment of the neuromuscular function in MG is a result of changes in the physiology, biochemistry and structure of the NMJ.

Physiology

At the nerve terminal, ACh is stored in synaptic vesicles. Occasionally a vesicle undergoes spontaneous exocytosis so that a so-called quantum of released ACh, consisting of about 10,000 molecules, causes a miniature endplate potential (MEPP), about 1 mV in amplitude, by interaction with the AChRs. MEPPs occur in the human endplate at a frequency of a few per minute and their amplitude is a good measure of the density of the AChRs.

When a nerve action potential reaches the nerve terminal, P/Q-type calcium channels open and as a result of inflowing calcium ions a number of vesicles release their ACh into the synaptic cleft. The evoked release of ACh is thus dependent on the presence of calcium in the surrounding medium. The number of ACh quanta released by the nerve action potential is called the quantal content in human muscle around 30 whereas in rodents it is between 50 and 100.

The ACh that is released upon a stimulus (in man 30x10,000 = 300,000 molecules of ACh) causes a depolarization called the endplate potential or EPP. The EPP has a more or less constant value since the nerve action potential is a standard event causing a standard inflow of calcium into the nerve terminal. The EPP shows a little variation, however, which is largely dependent on the variation in the quantal content and the variation in diameter of the muscle fiber.

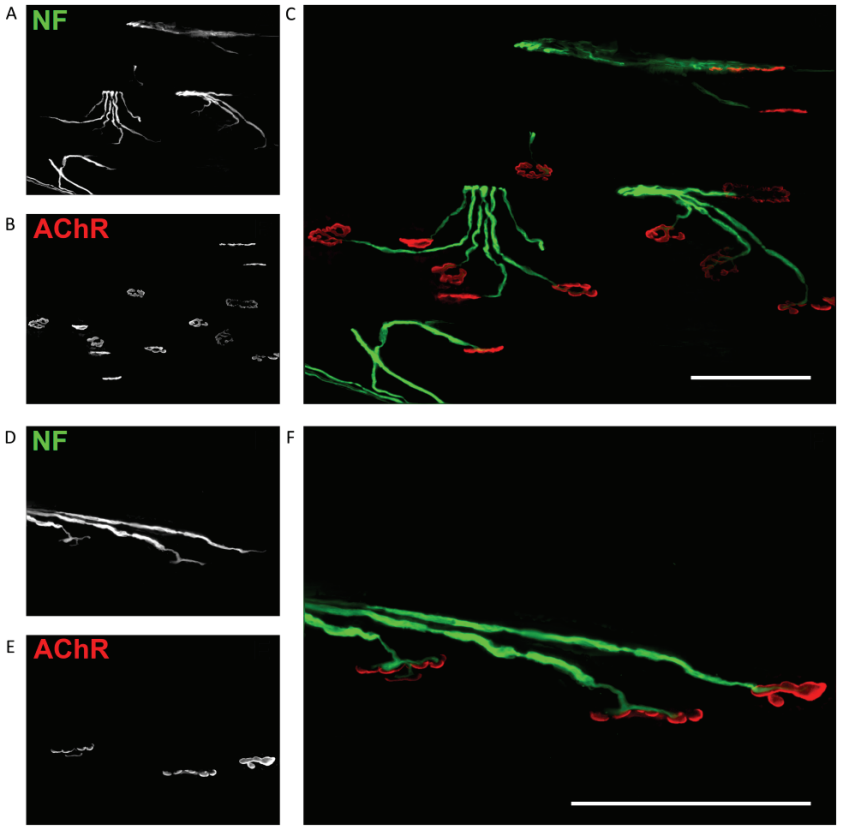

Figure 1.1. Immunohistochemical staining of 200 µm cryosections of rat tibialis anterior muscle. Nerves are stained with mouse anti-neurofilament Ab (A, D) and AChRs are stained with Alexa 594 α-bungarotoxin (B, E). Merged pictures (C, F) show the AChR clusters (red) at the site of contact with the nerve terminals (green). Generally, each muscle fiber is only innervated by a single nerve branch. Scale bars are 100 µm.

Normally, the EPP is so large that surrounding voltage-gated sodium channels (VGSCs) in the muscle membrane near the endplate are activated so that the muscle action potential is generated [16]. As soon as the action potential is generated, it completely overshadows the EPP (Fig. 1.4). If EPPs have to be recorded, this can only be done after blocking specifically the sodium channels of the muscle. Neuromuscular transmission has a large so-called safety factor, meaning that the EPP is considerably larger than the threshold membrane potential for opening of VGSC, so that nerve stimuli elicit muscle action potentials with 100% efficiency. This safety factor is about 3 in human muscle and about 5 in rodents [17].

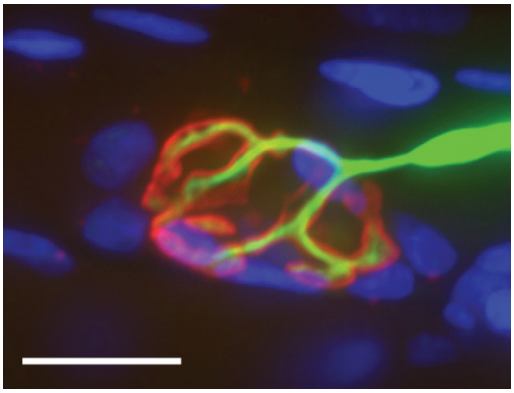

Figure 1.2. High power confocal photomicrograph of a rat NMJ. The AChR is stained in red with Alexa 594-labelled α-bungarotoxin and the nerve terminal is stained in green (Alexa 488) with an antibody directed against neurofilament and synaptic vesicle protein 2 (SV2). Nuclei are labeled with Hoechst DNA stain in blue. The image was taken with a MBF Bioscience Stereo Investigator Confocal Spinning Disk (SI-SD) system (MBF Bioscience, Williston, VT), with a modified Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan). Scale bar is 20 µm.

The muscle action potential spreads along the muscle fiber from the endplate area towards the tendons. The action potential activates L-type calcium channels causing the release of calcium from intracellular stores though ryanodine-type calcium channels. As a result, the concentration of cytosolic calcium rises dramatically, which leads finally to the contraction of the muscle fiber.

Biochemistry and structure

The nerve terminal produces agrin and neuregulin which induce expression of postsynaptic proteins in subsynaptic nuclei (Fig. 1.2 and 1.3) of the muscle fiber. Among the molecules that are concentrated at the endplate are the acetylcholine esterase, the membrane receptors for agrin [MuSK and low-density lipoprotein receptor-related protein 4 (Lrp4)] and the cytoskeletal-associated proteins utrophin and receptor-associated protein of the synapse (rapsyn) [18]. During development, AChRs are diffusely distributed on embryonic myotubes but become highly concentrated (approximately 10,000/μm^2) in the postsynaptic membrane a few weeks after birth, whereas the density of the extra-synaptical AChRs falls to ~10/μm^2 [19].

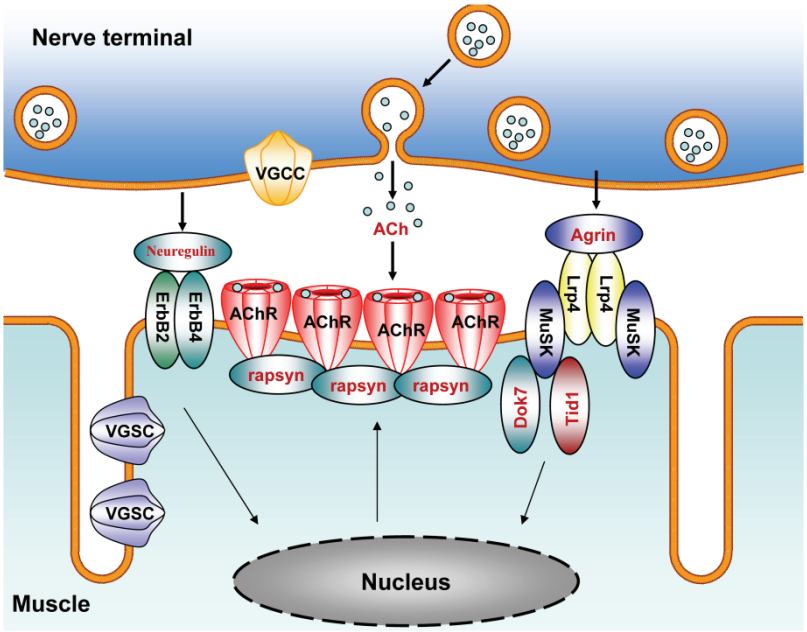

Figure 1.3. Scheme of the NMJ. ACh, neuregulin and agrin are released from the nerve terminal. ACh is a neurotransmitter that binds and opens the AChR ion channel, inducing depolarization of the membrane. Neuregulins bind to ErbB receptors and enhance the transcription of AChR, utrophin and acetylcholine esterase genes at the postsynaptic nuclei. Lrp4-MuSK-Dok-7-Tid1 are the main mediators of agrin-signaling in the postsynaptic membrane, activating intracellular cascades that lead to dense AChR clustering at the top of the junctional folds. ACh, acetylcholine; AChR, acetylcholine receptor; Dok-7, downstream of tyrosine kinase 7; ErbB2/4, erythroblastic leukemia viral oncogene homolog 2/4; Lrp4, low-density lipoprotein receptor-related protein 4; MuSK, muscle specific tyrosine kinase; rapsyn, receptor-associated protein of the synapse; Tid1, tumorous imaginal disk 1; VGSC, voltage gated sodium channel; VGCC, voltage gated calcium channel.

At least two distinct processes contribute to this accumulation. First, subsynaptic muscle nuclei transcribe AChR subunit genes at higher rates than extra-synaptic nuclei, so AChR messenger RNA is concentrated near synaptic sites [20, 21]. Second, once AChRs have been inserted into the membrane, they form high-density clusters by tethering to a subsynaptic cytoskeletal complex. A key component of this complex is rapsyn, a 43 kDa membrane-associated protein, which is precisely colocalized with AChRs at synaptic sites (Fig. 1.5) as soon as clusters form [22]. Rapsyn anchors the AChR via ß-dystroglycan and utrophin to the contractile protein F-actin and is thus essential for the AChR clustering in the endplate region [23-28]. No AChR clusters are formed in muscles of rapsyn-deficient mice or in myotubes cultured from the mutants [22].

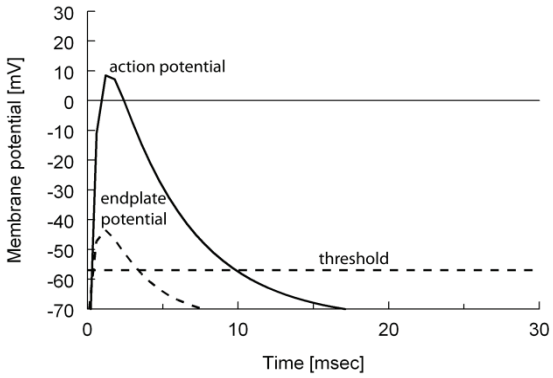

Figure 1.4. Schematic representation of an intracellular microelectrode recording at the endplate of the muscle action potential. The stippled line indicates the shape of the EPP (which can only be seen after blocking the action potential of the muscle with μ-conotoxin). The firing threshold of the muscle is indicated by the horizontal stippled line. Nota bene: in most recordings the electrode is dislodged from the cell because of the contraction of the muscle, but sometimes it stays put and it is possible, as illustrated in this chart, to follow the whole event of the muscle action potential.

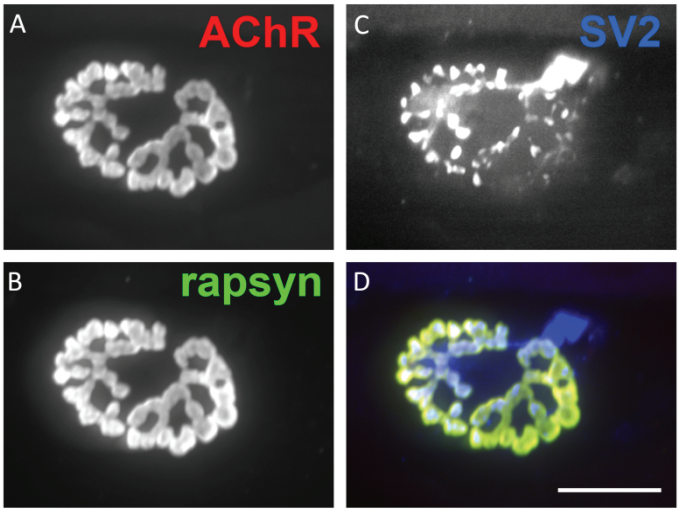

Figure 1.5. Confocal photomicrographs demonstrating colocalization of rapsyn and the AChR. Rat tibialis anterior cryosections of 30 μm were stained with Alexa 594 labeled α-bungarotoxin for the AChR in red (*A*), mouse anti-rapsyn mAb 1234 in green (*B*) and SV2 for the nerve terminal in blue (*C*). The merged picture (*D*) shows the precise colocalization of rapsyn and the AChR (yellow color). Images were taken with a MBF Bioscience Stereo Investigator Confocal Spinning Disk (SI-SD) system (MBF Bioscience, Williston, VT) with a modified Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan). Scale bar is 20 μm.

The structure of the postsynaptic membrane is also highly specialized. The nerve and the nerve terminal area are covered by Schwann cells as shown in the scanning electron micrograph in Fig. 1.6*A*. Chemical removal of the Schwann cell and partial removal of the nerve (Fig. 1.6*B*) reveal that the branched nerve endings are embedded in the gutter-like depression of the muscle plasma membrane (sarcolemma). Between the nerve endings (synaptic boutons) and the sarcolemma lies the primary synaptic cleft. Adjacent to synaptic boutons, the sarcolemma is folded and thereby creates secondary clefts (hereafter referred to as synaptic folds of the postsynaptic membrane, Fig. 1.6*B*, arrowhead). The postsynaptic folds enlarge the surface and contain a high density of AChR at the top of the folds facing the nerve and voltage gated sodium channels in the troughs of the secondary folds.

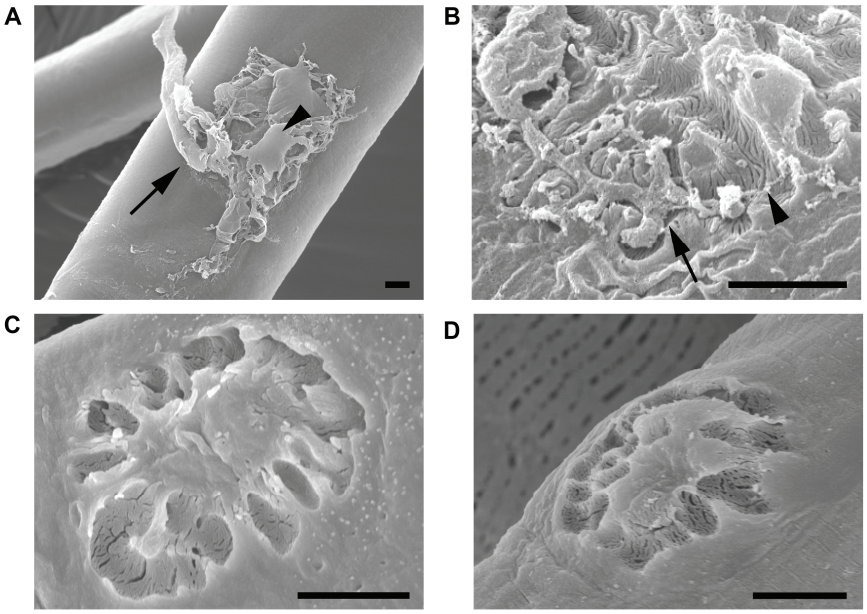

Figure 1.6. Scanning electron micrographs of the NMJ of a rat. (*A*) The myelinated motor axon (arrow) branches at the NMJ. The NMJ is covered by perisynaptic (terminal) Schwann cells (arrowhead). (*B*) Chemical removal of the Schwann cells exposes the nerve terminals (arrow) which are embedded in gutter-like depressions. Where the nerve terminal is removed as well, the folded postsynaptic membrane is visible (arrowhead). (*C*) Complete chemical removal of the presynaptic structures reveals the postsynaptic structure of a normal rat NMJ. (*D*) Postsynaptic membrane with slightly widened postsynaptic clefts in a rat with EAMG. Scale bars are 5 µm.

Pathophysiology of the neuromuscular junction in AChR-MG

Anti-AChR antibodies induce the loss of the AChRs, leading to impaired neuromuscular transmission with muscle weakness as a result [29]. Due to the reduced number of AChRs, the sensitivity of the endplate to released ACh is reduced. Consequently, the amplitude of the MEPPs is reduced with approximately the same proportion as the loss of the AChRs. However, the loss of AChRs is partly compensated by an increase in ACh release in AChR-MG [30, 31].

As a consequence of the reduced AChR levels in MG, the EPPs can be so low that the threshold for activating VGSCs is not reached and consequently no action potential is generated (blocking of neuromuscular transmission; Fig. 1.7*D*). If the EPP is just above the threshold, a delayed action potential is produced because the threshold is reached later. The reduced AChR levels, in combination with the variability of the quantal content, leads to a variation of the time at which an action potential is generated after motor nerve stimulation. This variation (jitter) can be measured by single fiber electromyography and is a sensitive measure for impaired neuromuscular transmission (Fig. 1.7*B*). This method also allows the detection of neuromuscular blockings.

The quantal content decreases as a function of time (and frequency) during repetitive stimulation. Due to the great safety factor of the NMJ, this does not normally impair neuromuscular transmission; but in MG, because of the decreased sensitivity of the endplate to ACh, this decreased output leads to an increased likelihood of neuromuscular blockings. In electromyography recordings in MG patients, the decreasing amount of released ACh during repetitive nerve stimulation (generally measured at 3 Hz) leads to increased number of neuromuscular blockings and to decrementing amplitudes of the compound muscle action potentials.

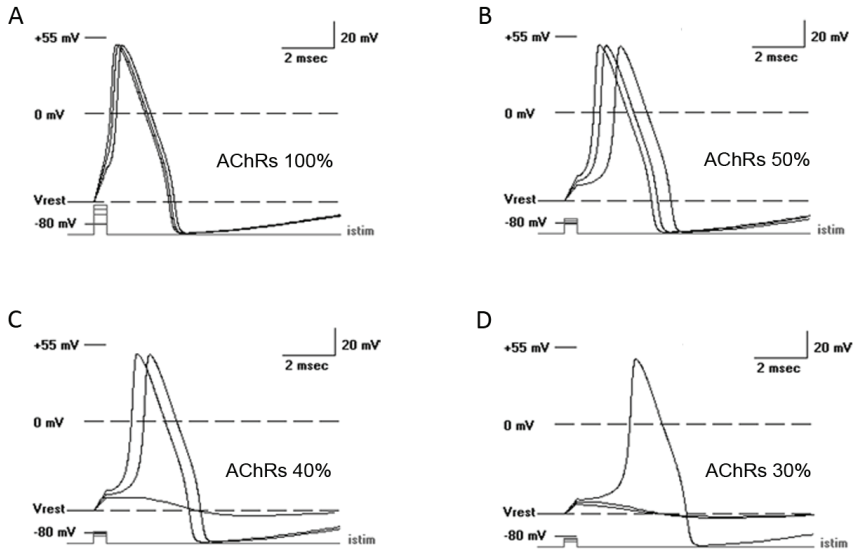

Figure 1.7. Computer simulation of muscle action potentials. Action potentials were elicited by nerve stimulation under conditions of increasing degree of AChR loss using the program NeuralSim/APSimv1.0 by Steven A. Siegelbaum. The basis of the simulation settings is the assumption of the value of the safety factor 3.0 for neuromuscular transmission in man. In each frame, (*A*)-(*D*), three traces are shown to indicate the variability of the quantal output of ACh upon each stimulus: the mean; the mean minus 1SD; and the mean plus 1SD (the coefficient of variation of the transmitter release of the human endplate with a quantal content of 30 is 0.18). Panel *A*, 100 % AChRs. Panel *B*, 50% AChRs; notice the increased delay in response time to the nerve stimulus and also the greater variation of response time (jitter). Panel *C*, 40% AChRs; notice the further delay in response time and one failure (blocking) where stimulation failed to elicit an action potential. Panel *D*, 30 % AChRs; a further delay in response time and two failures.

Ultrastructural changes at the NMJ in AChR-MG

In transmission electron micrographs, the ultrastructure of the postsynaptic membrane can be analyzed in detail. Normal NMJs show a complex pattern of postsynaptic folds near the nerve bouton (Fig. 1.8). In contrast, a high concentration of anti-AChR antibodies causes lysis of the postsynaptic membrane. Fig. 1.9*A* shows this lysis after passive transfer of the anti-AChR monoclonal antibody (mAb) 35. In this model, also infiltration of granulocytes occurs (Fig. 1.9*B*). Ultimately, the chronic exposure of the NMJ to anti-AChR antibodies leads to a simplified postsynaptic membrane without postsynaptic folds. This change also occurs in experimental autoimmune MG (EAMG) models (Fig. 1.9*C*), as discussed below.

Pathophysiology of the NMJ in MuSK-MG

The main effect of MuSK antibodies at the endplate is still unclear. MuSK is known to initiate aggregation of the AChR during synapse formation via the agrin/Lrp4/MuSK/rapsyn/AChR clustering pathway (Fig. 1.3), but MuSK is also expressed at the mature NMJ. MuSK autoantibodies have the potential to alter MuSK function at the adult NMJ, and they may not only inhibit MuSK function directly, but also increase the turnover of MuSK, thereby further reducing its activity [2, 3]. In contrast with AChR antibody-positive patients, there is no evidence of loss of junctional folds, no apparent loss of AChR density, and generally no complement deposition [32, 33] in muscle biopsies of MuSK-MG patients. These findings suggest that MuSK-MG may be different in etiological and pathological mechanisms, compared to AChR-MG [34].

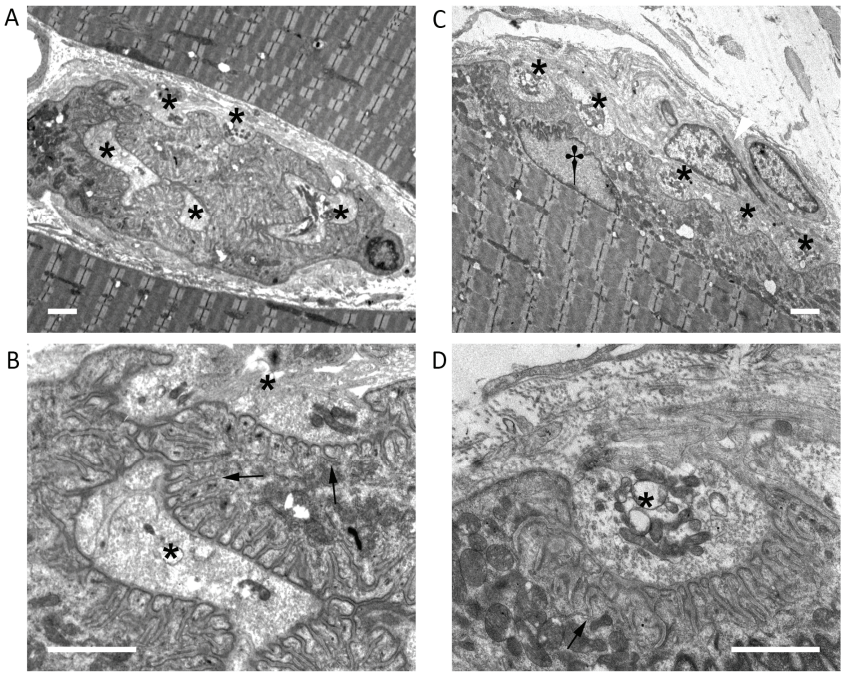

Figure 1.8. Transmission electron micrographs of the NMJ in rat tibialis anterior muscles. The asterisks indicate nerve terminals, the arrows point at the postsynaptic membrane (*A, B*) Section cutting the NMJ parallel to the muscle fiber surface (i.e. similar to the orientation of the scanning electron micrographs shown in Fig. 1.6). Abundant postsynaptic membrane folds are arranged around multiple nerve boutons. (*C, D*) Section cutting parallel to the axis of the muscle fiber. In this orientation, both the subsynaptic nuclei (dagger) and the perisynaptic Schwann cell (arrowhead) covering the nerve terminal. Scale bars are 2 µm.

Although some muscles may remain unaffected, other, particularly facial, muscles show abnormalities such as increased jitter and atrophy [35-37]. *In vitro* investigations revealed reduced amplitudes of MEPPs but no concomitant reduction in the number of AChRs [32, 33]. Studies using active immunization with MuSK or passive transfer of anti-MuSK antibodies from MuSK-MG patients to mice have shed some light into the effects on the NMJ and are discussed below.

IgG isotypes in MG

MG is mediated by circulating antibodies of the IgG class directed to the AChR, MuSK, or possibly other, still unidentified autoantigens in the NMJ of striated muscle. In humans, four different IgG isotypes exist which have similar amino acid sequences (more than 95 % sequence homology), but differ in their ability to activate the complement system. These differences are mainly due to structural differences at the CH_2 domains (the site of C1q binding) and variability of the hinge regions [38, 39]. IgG1 and IgG3 are effective complement activators, IgG2 poorly fixes complement, and IgG4 is completely deficient in the ability to activate complement via the classical pathway [40, 41]. The structure mainly responsible for the differential ability of human IgG isotypes to activate complement is located at the carboxy-terminal part (residues 292-340) of the CH_2 domain [42] (Fig. 1.10*A*), but the hinge region also has a minor contribution to the complement activation [43].

In AChR-MG patients, the complement-fixing IgG1 and IgG3 isotype AChR-specific antibodies predominate, whereas IgG2 and IgG4 are only present in lower concentrations [44-46]. Generally, the anti-AChR autoantibody concentration does not correlate well with the severity of disease [47], although in individual patients there is a relation between antibody titer and clinical condition after immunosuppression [48]. Since IgG autoantibody subclasses have distinct immunological properties, a possible explanation for this weak correlation could be found in variations in the isotype distribution. Interestingly, anti-AChR IgG1 (but not IgG2, IgG3, and IgG4) concentration was found to be significantly correlated with severity of disease [44]. These results suggest that binding of IgG1 may play a key role in the pathogenesis of AChR-MG [44]. In rhesus monkeys, passive transfer of human IgG1,

but not IgG4 anti-AChR antibodies, caused MG [49].

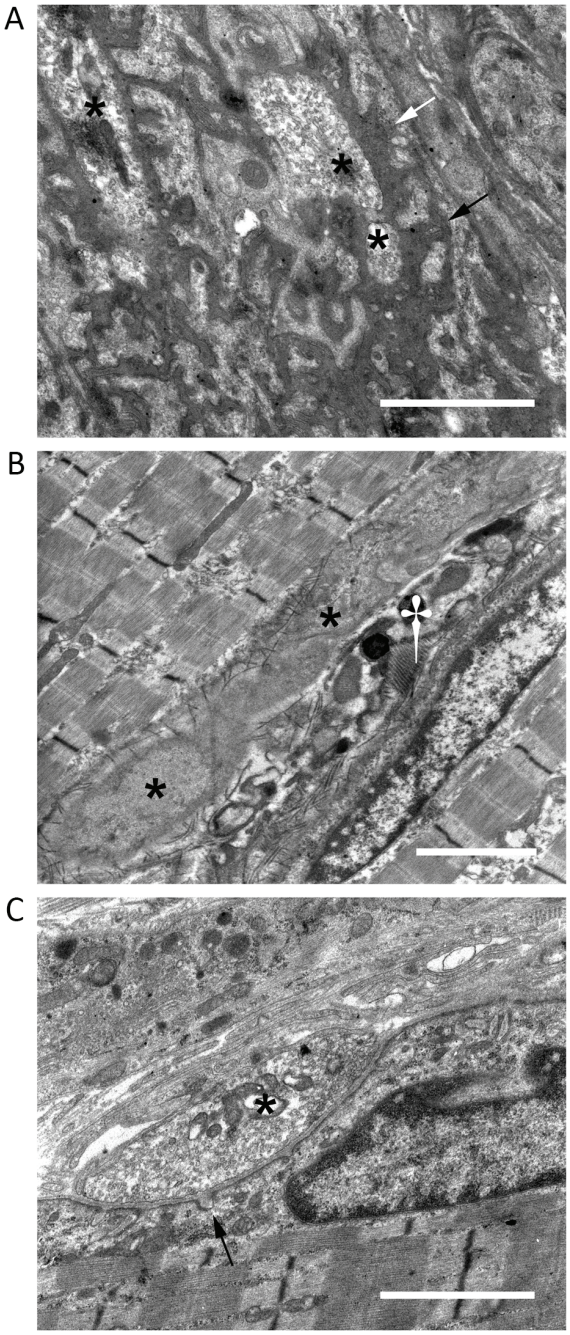

Figure 1.9. Morphological changes at the postsynaptic membrane after autoantibody attack. Transmission electron microscopy pictures of the NMJ in tibialis anterior sections. The asterisks indicate nerve terminals, the arrows point at the postsynaptic membrane. (*A*) Lysis of postsynaptic structure after passive transfer of anti-AChR mAb35. (*B*) Infiltrating granulocyte (see dagger) at the NMJ after passive transfer of mAb35. The synaptic boutons are dislocated from the sarcolemma. (*C*) In chronic AChR-EAMG, many endplates show a simplified postsynaptic membrane with a few shallow postsynaptic folds. Scale bars are 2 μm.

In contrast to the anti-AChR antibodies, anti-MuSK antibodies are predominantly of the IgG4 isotype [34] and therefore they do not cause substantial complement deposition, morphological damage, or AChR loss at the NMJ [32, 33]. These findings imply important differences between MuSK-MG and AChR-MG in their pathological mechanisms. The mechanism in MuSK-MG may involve downstream changes in the function and distribution of key molecules at the NMJ. Based on cell culture experiments using AChR-expressing rhabdomyosarcoma cells, it has been suggested that the postsynaptic machinery becomes structurally and functionally disorganized by a significant reduction of AChR clustering [50].

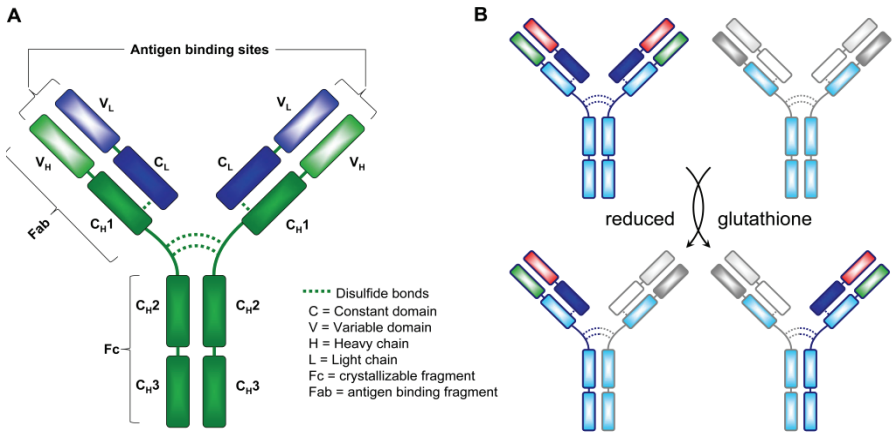

Figure 1.10. *A*) Structure of IgG. *B*) Fab arm exchange reaction. IgG4 molecules with different specificity interchange half-molecules resulting in chimeric bispecific antibodies [156].

Finally, in idiopathic MG patients, it has been shown that the autoantibodies belong to the IgG1 subclass. In addition, they induced complement deposition on the AChR clusters, which demonstrated C1q binding and activation of the classical complement pathway [13]. This suggests that the autoantibodies of idiopathic MG can be directed toward the AChR, but bind only when the AChRs are clustered densely [13].

IgG4 Fab arm exchange

All IgGs are composed of two heavy chain/light chain pairs (half-molecules), which are connected via inter-heavy chain disulfide bonds situated in the hinge region (Fig. 1.10*A*),

as well as by non-covalent bonds mostly situated between the third constant domains (CH_3). IgG antibodies mediate pro-inflammatory activities, with the exception of IgG4 which has anti-inflammatory activities. IgG4 represents approximately 4% of the total IgG in serum of adults and significant IgG4 titers are generated by prolonged antigenic stimulation [51].

The anti-inflammatory activity includes a poor ability to induce complement and cell activation because of a low affinity for C1q and Fc receptors. Moreover, IgG4 does not form immune complexes due to a posttranslational modification, known as Fab arm exchange. IgG4 exchanges Fab arms by swapping a heavy chain and attached light chain with a half-molecule from another antibody (Fig. 1.10*B*). Antibodies from the IgG4 subclass have been shown to be dynamic molecules, undergoing Fab arm exchange both *in vivo* and *in vitro*. The ability to engage in Fab arm exchange appears to be an inherent feature of IgG4 that involves the third constant domain in addition to the hinge region and this posttranslational modification only requires a reducing environment to be activated [49].

Kinetic studies on the inter-heavy chain disulfide bond formation of the IgG4 molecule showed that these bonds were formed slowly and that they were unstable [52]. Inter- and intra-heavy chain disulfide bonds are in equilibrium. This phenomenon is facilitated by a substitution of single amino acid in the hinge of IgG4 compared to IgG1: a proline in IgG1 is replaced by a serine in IgG4 [53]. Nevertheless, simply mixing IgG4 molecules *in vitro* does not result in Fab arm exchange. The mechanism by which IgG4 Fab arm exchange occurs *in vivo* likely requires the reducing environment in blood or at cell surfaces to facilitate the breaking of bonds between half-molecules [49].

When polyclonal IgG4 is derived from plasma, the Fab arm exchange reaction usually produces IgG4 molecules with two different antigen-binding sites. The resulting asymmetric antibodies are bispecific, and thus are directed against two different and generally unrelated antigens. Moreover, they are unable to cross-link two identical antigens [54] and are therefore functionally monovalent [51]. Fab arm exchange, furthermore, is dynamic, and combinations of certain specific Fab arms are therefore expected to exist only transiently. This protein modification challenges the commonly

accepted one antibody-one antigen paradigm and redefines our thinking about the role of IgG4 in antibody-mediated immunity and the application of IgG4 monoclonal antibodies to immunotherapy.

Functionally monovalent IgG4 antibodies directed to the AChR that do not modulate the AChR or block the ACh binding are not pathogenic. Moreover, they have the potential to protect the NMJ against complement fixing IgG1 anti-AChR-autoantibodies [49] by competition for binding. Therefore, IgG4 has an important anti-inflammatory property and protects tissues against the biological effects of the complement-fixing IgG subclasses [54].

Effector functions of IgG autoantibody subclasses in AChR-MG

The breakdown of the AChR is mainly caused by complement-mediated lysis of the postsynaptic membrane [55] and by cross-linking of AChRs in the membrane. Cross-linking antibodies are believed to initiate a conformational change of the AChR (antigenic modulation) which induces accelerated AChR internalization and degradation [56]. The different properties and effector functions of IgG antibodies are discussed below.

Antigenic modulation

Antibodies from patients with AChR-MG accelerate the degradation of AChRs in cultured muscles and *in vivo* at intact NMJs [56-58]. The antibody-accelerated degradation of the AChR by antigenic modulation is a consequence of the bivalent nature of the IgG1, IgG2 and IgG3 autoantibodies. These AChR-specific antibodies are able to cross-link adjacent AChR molecules which are rapidly internalized by endocytosis and then degraded [59]. Serum IgG from approximately 90% of patients increased the degradation rate of the AChR two to threefold [60]. Thus, if accelerated degradation is not sufficiently compensated by increased AChR synthesis, it will lead to a reduction of the available AChR molecules at the NMJ. This reduction of AChR at the NMJ can be used as a useful diagnostic test for MG [61]. However, not all anti-AChR antibodies cause antigenic modulation. The epitope location on the AChR surface may restrict the ability of antibodies to cross-link a second AChR molecule [62]. Moreover, IgG4 antibodies are

functionally monovalent, which means that they do not cross-link two identical antigens.

Complement activation

Binding of anti-AChR antibodies to the tightly packed AChRs in the postsynaptic membrane folds results in very high density of bound antibody and, hence, very tightly packed Fc regions of these antibodies. Since anti-AChR antibodies belong mainly to the IgG1 and IgG3 class, this causes a highly efficient activation of the complement system and consequently the formation of the membrane attack complex (MAC) in the postsynaptic membrane. In combination with antigenic modulation of the AChR, MAC causes severe endplate membrane damage [63, 64]. Stabilization of the AChR by increasing rapsyn expression prevents modulation of the AChR, even in the presence of activated complement [65, 66].

Different lines of indirect evidence support complement activation at the NMJ as a fundamental cause of AChR loss and failure of neuromuscular transmission: complement depletion with cobra venom factor [67], administration of antibodies that block a complement component [68], complement inhibitors [69], or genetic deficits of complement components [70] make animals resistant or less susceptible to EAMG. IL-12 deficient mice develop minimal EAMG symptoms after AChR immunization in spite of robust anti-AChR antibody synthesis [71]. The lack of IL-12 prevents the production of the complement-fixing IgG2a antibodies in the mouse (Table 1.2). NMJs in these mice contain only IgG1 antibodies, which unlike human IgG1 antibodies do not activate complement; indicating that anti-AChR antibodies, which do not activate complement, do not effectively compromise neuromuscular transmission.

Complement activation damages the postsynaptic membrane by multiple mechanisms. The MAC leads to loss of postsynaptic folding [72], loss of membrane potential [73] and, in conjunction with antigenic modulation of the AChR, to loss of AChR-associated proteins [74].

Functional AChR blockade

Serum IgG from 50 to 88% of patients with MG has been shown to block the ACh-binding sites of AChRs in cultured mammalian muscle cells [61]. Antibodies with the ability to

block the ACh-binding site of the AChR cause acute and severe muscle weakness in rodents without inflammation or necrosis at the NMJ [75]. Many MG patients have low levels of these antibodies. They might block the AChR in spite of their low concentrations and contribute to acute myasthenic crisis [76].

Loss of AChR-associated proteins

The attack of anti-AChR antibodies at the NMJ does not only lead to a reduction in the density of AChR, but also affects other AChR-associated proteins [77, 78]. Research on the significance of postsynaptic proteins for the maintenance of the adult endplate and the reorganization of the NMJ after injuries are promising, showing that some postsynaptic proteins are not just crucial for fetal development but also play an important role in the dynamics of the organization of adult endplates. This premise is in agreement with the deterioration of neuromuscular transmission observed in patients with mutations in genes of the NMJ. This group of patients is gathered under the term “congenital myasthenic syndromes” (CMS) [79]. CMS mutations can occur in genes of presynaptic, synaptic, or postsynaptic proteins, though the latter is far more common [80].

The importance of the synaptic proteins is further illustrated in the knock-down models for MuSK [81], rapsyn [22], Dok-7 [82], Lrp4 [83], or agrin [23], where animals die prematurely around birth, primarily due to respiratory distress, and with a severely underdeveloped NMJ.

Among the NMJ proteins affected in CMS patients, the AChR is the most prominent, with mutations in α, β, δ or ε subunits that generate an MG-like clinical picture of the patients. Several mutations that lead to muscular weakness have been identified in rapsyn, MuSK, laminin and Dok-7 genes [84-88]. CMS patients display a variable range of MG-like symptoms that are normally present at birth or develop during early life.

In AChR-MG, it seems likely that loss of AChR-associated proteins, including rapsyn, utrophin and voltage gated sodium channels aggravate the disease and delays repair processes [74, 89]. The function of key postsynaptic proteins is reviewed below, because we believe that this knowledge is helpful for evaluating the possible consequences resulting from loss of these proteins in AChR-MG.

Table 1.2. IgG isotype profile and complement activation in relation to T helper subsets and cytokine production.

	Mouse		
Isotype	IgG1 (1)	IgG2a (2)	IgG2b (2)
Complement activation	No	Yes	Yes
Cytokines involved in class switching and IgG production	IL4 (a,b) IL5 (b) IL6 (b) IL10 (b)	IL2 (b) IFNγ (a,b) IL12 (d)	TGFβ1 (a)
Th control and effector function	Th2 Anti-inflammatory	Th1 Pro-inflammatory	Pro-inflammatory
	Rat		
Isotype	IgG1 (1)	IgG2a (1)	IgG2b (2)
Complement activation	Yes	Yes	Yes
Cytokines involved in class switching and IgG production	IL4 (c) IL10 (f)		IL2 (c) IFNγ (c) IL12 (c)
Th control and effector function	Th2 Pro-inflammatory	Pro-inflammatory	Th1 Pro-inflammatory

	Human			
Isotype	IgG1	IgG2	IgG3	IgG4
Complement activation	Yes	No	Yes	No
Cytokines involved in class switching and IgG production	IL2 (g) IL4 (a) IL6 (g) IL10 (a) IL21 (e)	IL2 (g) IFNγ (h)	IL2 (g) IL4 (a) IL6 (g) IL10 (a) IL21 (e)	IL2 (g) IL4 (a) IL6 (g) IL12 (i) IL13 (a)
Th control and effector function	Pro-inflammatory	Anti-inflammatory	Pro-inflammatory	Anti-inflammatory

(1) Mouse IgG1 is homologous to rat IgG1 and rat IgG2a [149]. (2) Mouse IgG2a and mouse IgG2b are homologous to rat IgG2b [149]. (a) [150]; (b) [151]; (c) [127]; (d) [129]; (e) [152]; (f) [153]; (g) [154]; (h) [155]; (i) [156].

Utrophin

Utrophin is localized primarily at the NMJ [90], while the homologous protein dystrophin [91] is expressed throughout the muscle fiber [92]. Utrophin is found closely colocalized to the AChR both in the adult NMJ [93] and throughout development [94], suggesting that it helps to stabilize the AChR clusters [95]. However, studies of utrophin-deficient mice

indicate that utrophin is not essential for NMJ formation or for precise localization of AChRs at the NMJ [96, 97]. The reduction of utrophin in MG patients is probably secondary to the loss of AChR, but in the presence of autoantibodies against the AChR, it is likely that utrophin-loss curbs anchoring of newly produced AChRs to the cytoskeleton [74].

Rapsyn

Rapsyn is required for anchoring and stabilizing the AChR in the postsynaptic membrane of the NMJ during development. The AChR clustering by rapsyn is activated by agrin [98], which acts via Lrp4 and MuSK [99]. The expression of rapsyn increases with age and thereby stabilizes the AChR [100]. In a passive-transfer rat model for MG, increased expression of rapsyn at the NMJ induced resistance against anti-AChR antibodies [66] by reducing antibody-induced AChR internalization. Conversely, rapsyn overexpression has a detrimental effect in chronic EAMG where endplates are already substantially damaged. In chronic EAMG, increased rapsyn expression increases the postsynaptic membrane turnover by anti-AChR antibodies [74]. It is of interest that a modest reduction in rapsyn expression already causes substantial changes, showing that the amount of rapsyn is critically related to the AChR levels and to the structure of the endplate [89].

These findings support the idea of rapsyn as a stabilizing protein for the AChR in the membrane, as previously shown *in vitro* when AChR half-life was increased and its degradation rate reduced by the presence of rapsyn in cultured myotubes [28], or when AChR turnover was augmented in rapsyn-deficient myotubes [27]. In intercostal muscle biopsies from AChR-MG patients rapsyn levels are significantly reduced relative to control biopsies (unpublished results).

MuSK

The role of MuSK has been discussed above. In addition it is important to mention the findings of Hesser et al. [101] who demonstrated the relevance of MuSK for the maintenance of the postsynaptic structure using a transgenic mouse model with a conditional knock-out of MuSK. Upon inactivation of MuSK expression, the AChR clusters became disassembled with evident disorganization of the NMJ and sprouting of the nerve

terminals. This further supports the notion that MuSK expression is necessary for maintaining the integrity of the NMJ [4].

Lrp4

Lrp4 is localized in the postsynaptic membrane and has a large extracellular region [102, 103]. New born mice with null alleles of Lrp4 are cyanotic, unable to breathe, and have defects in distal limbs, indicating that Lrp4 is required for the development of the NMJ [83]. The absence of AChR clusters at the postsynaptic membrane of Lrp4 null mutant animals resemble the phenotype observed in MuSK -/- mice and suggest a role for Lrp4 upstream or at the same level of MuSK. Lrp4 selectively binds to neural agrin [99, 104], which is necessary for the activation of MuSK.

Dok-7

The family of Dok proteins works as docking platforms for the formation of signaling complexes at the cell membrane. They actively regulate signal transduction and recruit other proteins by their phosphotyrosine binding (PTB) domain and a C-terminal domain, which contains multiple potential tyrosine phosphorylation sites and proline-rich sequences for SH2 and SH3-containing proteins [105]. Dok-7 interacts with the cytoplasmic domain of MuSK and induces its autophosphorylation. MuSK-deficient myotubes do not form AChR clusters when mutated forms of MuSK, which cannot interact with Dok-7, are reintroduced [82]. Silencing of Dok-7 in C2 myotubes suppressed MuSK phosphorylation and, therefore, impaired the formation of AChR clusters. Dok-7 knock-out mice are immobile and die at birth. No detectable AChR clusters on the endplate area are developed in these mice, which resembles the phenotype of MuSK -/- mice [82].

Tid1

When screening for proteins that interact with MuSK, Linnoila and colleagues [106] encountered a rat homolog of the *Drosophila* tumor suppressor Tid56 and the heat shock protein hsp40 that was constitutively associated with MuSK, and was denominated tumorous imaginal disk 1 (Tid1). Tid1 is colocalized with MuSK and the AChR at the postsynaptic membrane. The relevance of Tid1 for AChR clustering was demonstrated

when silencing of Tid1 expression in cultured myotubes caused a marked disruption in the AChR clusters, a situation that could be rescued by transfecting the normal Tid1 gene [106]. However, transfection of a mutated variant of Tid1 did not rescue AChR clustering. These findings suggest that Tid1 orchestrates the cascade that leads to AChR clustering by interactions with other regulatory proteins downstream of MuSK, since Tid1 had been documented to activate a variety of intracellular mediators like the small GTPases RAc1 and RhoA [107] or NFκB [108]. Some of these mediators were directly related with AChR phosphorylation and rapsyn stabilization like Hsp90β [109] and, therefore, it is likely that the effect of Tid1 on the AChR clustering occurs downstream of MuSK [106, 110].

In vivo studies have also supported the role of Tid1 for the development and maintenance of the NMJ. Tid1 knock-out mice die at embryonic day 7. Since Tid1 is localized in many cells types and tissues [111] and is involved in a wide range of intracellular processes such as DNA repair [112] and senescence [113] or signaling pathways like those of NF-κB [108], interferon [114] and *ras* [107], it is not surprising that suppression of this gene resulted in death at an early developmental stage. Furthermore, focalized siRNA-mediated downregulation of Tid1 in adult mouse muscles led to a profound disorganization of the AChR clusters at the NMJ and also impaired neuromuscular transmission, showing that Tid1 expression is essential for the maintenance of normal endplates [106].

Although MuSK still remains a key molecule for AChR clustering, it is more adequate at the moment to refer to a MuSK-Dok7-Tid1 complex in the agrin-induced pathway for synapse development.

IgG isotypes in animal models for MG

Animal models have contributed significantly to elucidate the pathogenesis of MG. AChR-immunization is used as an MG animal model termed EAMG. Moreover, injection of MG patient sera or monoclonal anti-AChR antibodies also induce muscle weakness in animals and this model is termed passive transfer MG [115].

Similarly, MG can be induced in animals by active immunization with MuSK [116, 117] or by passive transfer with serum from MuSK-MG patients [118, 119]. Here, we refer

to these models as “MuSK-EAMG” and “passive transfer MuSK-MG”, respectively (Table 1.1).

MG models have been crucial for understanding the role of the different IgG isotypes in the pathology and severity of MG. This knowledge provides a basis to improve the current therapies for MG patients and to develop more specific treatment approaches [120]. Strategies that use this knowledge to curb the pathogenic effects of pro-inflammatory IgG subclasses are briefly discussed next.

AChR-EAMG

Patrick and Lindstrom were the first to induce MG in rabbits by immunization with AChR from the electric organ of electric eels (*Electrophorus electricus*) in complete Freund’s adjuvant. They immunized these rabbits with purified AChR to obtain antibodies for the biochemical analysis of the AChR, but all animals became paralyzed and eventually died [121]. Active immunization of experimental animals (including mice, rats, guinea pigs, and monkeys) with AChR -with or without adjuvant- induced chronic EAMG within 30 days after immunization [122-124]. The animals mount an active immune response against injected AChR. The disease is caused by antibodies cross-reacting with the animals’ own muscle AChR.

The production of the pathogenic antibodies by B cells depends on T helper cells. The pathogenic anti-AChR antibodies are high-affinity IgGs, whose synthesis requires interaction of activated T cells with B cells [125]. T cells can be divided into T helper-one (Th1) and T helper-two cells (Th2) depending upon their cytokine profile. Cytokine signaling is crucial for development, modulation, and downregulation of immune responses, and therefore influences the initiation and evolution of the anti-AChR response in EAMG. The regulation of Th cells by cytokines is important, because it affects the isotype profile of stimulated B cells. Th1 cells have a pathogenic role in EAMG because they stimulate the synthesis of anti-AChR antibodies that fix complement and therefore cause destruction of the NMJ [126]. In contrast, Th2 cells induce anti-AChR antibodies that do not fix complement in mice (Table 1.2). In rats, both Th1 and Th2 cells are able to induce pathogenic anti-AChR antibodies [127].

In AChR-EAMG models, the isotype profile of autoantibodies can be altered by experimental treatments. Cytokines such as IL-4 stimulate Th2 cells and antagonize the action of Th1 cells, possibly by inducing regulatory T cells [128]. In mice, manipulation of the balance in favor of Th2 cells protects against EAMG [129], because only Th1-related cytokines induce the complement activating IgG2b antibodies. In rats, both Th1 and Th2 cells are able to induce pathogenic anti-AChR antibodies (see Table 1.2) and manipulation of the Th1/Th2 balance does not affect the severity of disease [127]. The different requirements for Th1-dependent responses in rats and mice are therefore correlated to the capacity of complement activation by antibody subclasses. In humans, it is not yet clear if the Th1/Th2 balance affects the production of complement-binding antibodies. It would be interesting to study how an isotype switch of IgG1 towards IgG4 can be induced in autoreactive B cells for the treatment of AChR-MG.

Passive transfer AChR-MG

Toyka and colleagues have transferred purified IgG from MG patients to mice, which subsequently developed MG symptoms [115]. This passive transfer EAMG model is relevant for MG in order to study the effector phase of the disease. Antibodies against the main immunogenic region (MIR) of the AChR induce myasthenia within 8 to 48 hours, depending on the dose and affinity of the antibody for AChR [130]. The source of antibodies can be serum of MG patients, serum from chronic EAMG animals, or monoclonal antibodies produced in cell culture [131]. The immunopathological mechanisms are antigenic modulation [132] and complement-mediated focal lysis of the postsynaptic membrane for anti-MIR antibodies [67]. Antibodies against the ACh-binding sites induce an acute paralysis within 15-30 minutes [133]. It has been shown that also anti-MIR antibodies can functionally inhibit AChR [134], and the role of the MIR for AChR activity has recently been demonstrated [135].

Monovalent anti-AChR antibodies or antibody fragments without complement binding capacity are not pathogenic. Since they can compete with pathogenic autoantibodies for binding to the AChR, they can prevent autoantibody binding at the NMJ [136]. In passive transfer AChR-MG mouse models, monovalent Fab fragments have been

demonstrated to protect the AChR against the action of intact pathogenic antibodies [137, 138]. Monovalent binding to antigens is not limited to Fab fragments, but also human IgG4 antibodies are functionally monovalent. IgG4 has favorable properties for potential immunotherapy, such as long half-life *in vivo*, low immunogenicity, no capacity of complement activation, and inability to cross-link antigens. In a passive transfer MG model in rhesus monkeys, a monoclonal human anti-AChR IgG4 antibody derived from an MG patient [139] was not pathogenic, while the corresponding IgG1 isotype induced muscle weakness [49]. Interestingly, also Fab arm exchange of IgG4 anti-AChR antibodies occurred in this model, suggesting that the rhesus monkey has an IgG4 like subclass.

MuSK-EAMG

Mice actively immunized with MuSK also showed the characteristic symptoms of MG [117]. However, the pro-inflammatory (i.e. complement-fixing and cross-linking) anti-MuSK antibodies that are produced in MuSK-EAMG do not closely resemble human MuSK-MG with anti-inflammatory IgG4 antibodies. Therefore, the pathological mechanisms of the MuSK-EAMG model might differ from MuSK-MG.

Passive transfer MuSK-MG

The presence of a pathogenic antibody in MuSK-MG was demonstrated by a passive transfer of IgG from anti-MuSK-positive MG patients into adult mice. In this passive transfer model, reduced levels of AChRs were observed at the postsynaptic membrane and changes in the presynaptic and postsynaptic elements of the synapse led to muscle weakness [118]. Treatment of mice with blood plasma from MuSK patients led to reduced endplate sizes and caused a decrease in the safety factor of neuromuscular transmission in regenerating adult muscles [119]. With high doses of anti-MuSK IgG in passive transfer experiments, the number of AChRs is reduced as well [118]. It is possible that MuSK antibodies in patients primarily cause a reduction in the size of the presynaptic ending while leaving the postsynaptic part and the AChRs intact, although the function of the AChRs may be altered [119]. In this case, the value of the quantal content would be decreased leading to increased jitter in single fiber EAMG. Reduced endplate sizes have been observed in pathogenic mutations of both MuSK and Dok7 [85, 86], which predict

that the pathophysiological cause of muscle weakness is primarily a small value of the quantal content because of small nerve terminals.

Conclusion

The symptoms of AChR-MG and MuSK-MG are a result of dysregulation of the immune system and the NMJ. Antigen-specific therapies for MG can make use of biological mechanisms such as the Th1/Th2 balance or the IgG1/IgG4 antibody isotype distribution. Similarly, the NMJ offers potential therapeutic targets to make the postsynaptic membrane more resilient to autoantibody attack. Hopefully, a better understanding of the mechanisms of immune regulation in MG and the pathogenesis at the NMJ as a result of autoantibody attack will lead to the development of antigen-specific therapies.

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Chapter 2

Proteomic analysis of rat tibialis anterior muscles at different stages of experimental autoimmune myasthenia gravis

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Abstract

Myasthenia gravis (MG) is an autoimmune disease in which autoantibodies, most commonly directed against the acetylcholine receptor (AChR), impair neuromuscular transmission and cause muscle weakness. In this study, we utilized two-dimensional difference in-gel electrophoresis (2D-DIGE) to analyze the muscle's proteomic profile at different stages of experimental autoimmune myasthenia gravis (EAMG). We identified twenty-two differentially expressed proteins, mainly related to metabolic and stress-response pathways. Interestingly, these identified proteins have also been associated with other contraction-impairing muscle pathologies (e.g. inclusion body myositis), suggesting a similar response of the muscle to such conditions.

Introduction

Myasthenia gravis (MG) is one of the best characterized antibody-mediated autoimmune diseases, and its symptoms include muscle weakness and fatigability. The autoantibodies in MG are primarily directed to proteins of the neuromuscular junction (NMJ), such as the acetylcholine receptor (AChR-MG, 85% cases), muscle-specific kinase (MuSK-MG, 10% cases) and low-density lipoprotein receptor-related protein 4 (Lrp4-MG, ~2% cases) [1, 2]. In AChR-MG, autoantibodies cause extensive damage at the NMJ, mainly by activation of the complement system and by antigenic modulation of the AChR [3]. This autoimmune attack leads to a reduction of AChRs and other AChR-related proteins [4, 5], which further contributes to the severity of symptoms. Consequently, such AChR-related proteins could represent novel targets for symptomatic treatments. However, the precise number and identity of muscle proteins affected by the autoimmune attack in MG remains largely unknown.

In recent years, the advent of the two-dimensional difference in-gel electrophoresis (2D-DIGE) technology has allowed a more sensitive and accurate quantification of differential protein expression and/or protein modifications affecting size and charge in biological samples [6]. In this study, we used the experimental autoimmune myasthenia gravis model (EAMG) rat model and 2D-DIGE to evaluate the muscle's proteomic profile at different disease stages of EAMG.

Materials and Methods

Animals, induction of EAMG and tissue preparation

Seven-week-old female Lewis rats were obtained from Charles River laboratories (Cologne, Germany). Chronic EAMG was induced by immunization with AChRs purified from *T. californica,* the severity of EAMG symptoms was assessed three times a week with the paw-grip test for muscle weakness as described [7]. Animals were sacrificed between 5 and 8 weeks after immunization according to their disease score, to have a

representative number of muscles from each score (0, no weakness; 0/1, mild weakness after testing; 1, weakness after testing; 2, clinical signs of EAMG present before testing; 3, severe clinical signs of EAMG, moribund). Animals were perfused to obtain blood-free tibialis anterior muscle [5 mM EDTA in PBS pH 7.2, containing Complete Protease Inhibitor Cocktail (Roche, Almere, the Netherlands)]. Muscles were processed to remove their connective membranes and tendons, frozen in liquid nitrogen and subsequently stored at -80°C. All experiments were done with permission from the Committee on Animal Welfare of Maastricht University, according to Dutch governmental rules.

Protein Extraction

Proteins were extracted as described [8]. Briefly, muscle samples were lyophilized, crushed (GentleMACS, Miltenyi Biotec, Leiden, the Netherlands) and proteins solubilized before ultracentrifugation. Samples were then desalted and the buffer was exchanged to labeling buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS in 30 mM Tris HCl pH 8.5) using Amicon Ultra filters (Millipore, 3 kDa cut-off). Protein concentration was determined using the 2D Quant kit (GE Healthcare, Diegem, Belgium) and aliquots were stored at -80°C. For Western blotting experiments, additional muscles from animals described in [7] were homogenized with a Mini BeadBeater (Biospec Products, Bartlesville, OK) in lysis buffer (30 mM triethanolamine, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM Na-orthovanadate, 1 mM benzamidine, 1 mM Na-tetrathionate, 1 mM PMSF, pH 9.5) containing HALT Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL) and Triton X-100 (1%).

2D-DIGE

Minimal labeling with N-hydroxysuccinimidyl-ester dyes Cy2, Cy3 and Cy5 (GE Healthcare, Diegem, Belgium) and 2D-GE were performed as described in [9]. CyDye-labeled 2D-DIGE gels were scanned on the Ettan DIGE imager (GE Healthcare). Gel images from all three CyDyes were loaded into DeCyder 7.0 software (GE Healthcare) and analyzed. Statistical significance was calculated using analysis of variance (ANOVA) and multiple comparison test. Spots present in 85% of the gel images, and with $p \leq 0.05$, were considered for further analysis.

Spotpicking, protein digestion and identification

For spot picking (ProPicII, Isogen Life Science, PW De Meern, The Netherlands) a 12.5% acrylamide gel was loaded with 200 µg of an unlabeled internal standard and 50 µg of the Cy2 labeled internal standard. In-gel digestion using trypsin (Promega, Leiden, the Netherlands) [10] was performed followed by protein identification by mass spectrometry [9].

Western blotting

To validate the results obtained by 2D-DIGE, we performed Western blotting both with muscle extracts used for the 2D-DIGE analysis and muscle extracts from control and EAMG animals characterized in a previous study [7]. Protein extracts were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and membranes were incubated with either rabbit anti-β-enolase (W-25, Santa Cruz Biotechnology, CA; 1:800) or goat anti-carbonic anhydrase III (CAIII) (E-19, Santa Cruz Biotechnology; 1:800), and mouse anti-GAPDH (10R-G109a, Fitzgerald, MA; 1:5000000). Secondary antibodies were donkey anti-mouse IgG IRDye 680, goat anti-rabbit IgG IRDye 800 and donkey anti-goat IgG IRDye 800 (926-32222, 926-32211 and 926-32214 respectively; LI-COR Biosciences, Lincoln, NE), diluted 1:10000. Membranes were imaged with Odyssey Infrared Imaging System (LI-COR Biosciences) and bands were quantified with ImageJ software (mean intensity), normalizing for GAPDH mean intensity. Protein levels were compared between groups with an unpaired *t*-test using GraphPad Prism 4 software.

Results

A 2D-DIGE proteomics experiment was performed to identify proteins that are affected at various disease stages of EAMG. Proteins were extracted from blood-free tibialis anterior muscles from each of the experimental groups (control, clinical scores 0, 0-1, 1, 2, 3; n = 4). A 2D spotmap with an average of 3342 protein spots per sample was obtained and analyzed in the DeCyder 7.0 software.

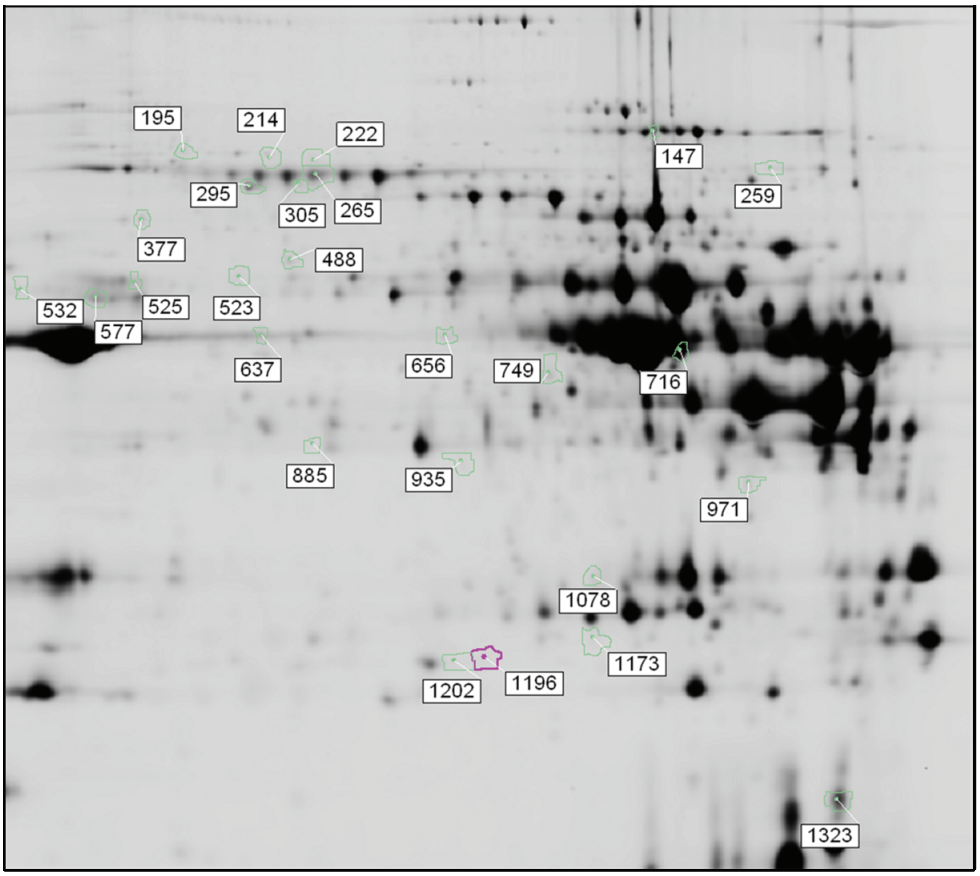

Figure 2.1S. 2D-DIGE gel image. The twenty-six protein spots that are differentially expressed in EAMG are shown (1w-ANOVA, $p \leq 0.05$). Seventeen of these spots were successfully identified by mass spectrometry (significant MASCOT and SEQUEST scores).

Table 2.1. Protein identification

Spot number	1w-ANOVA	Relative fluorescence intensity	Protein identification	Protein accession numbers
147	0.0233	decreased	Pyruvate kinase isozymes M1/M2	IPI00231929
			Serotransferrin	IPI00679202
			Aconitate hydratase, mitochondrial	IPI00421539 IPI00950672
195	0.0255	depends on experimental group	Myosin-4	IPI00476111 IPI00948572
			mitochondrial inner membrane protein	IPI00364895 IPI00566985 IPI00777695 IPI00948331
214	0.015	decreased	mitochondrial inner membrane protein	IPI00364895 IPI00566985 IPI00948331
265	0.00177	decreased	Serum albumin	IPI00191737
295	0.0318	decreased	Dihydrolipoylly sine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	IPI00231714
			Serum albumin	IPI00191737
305	0.000398	decreased	Serum albumin	IPI00191737
377	0.0131	decreased	60 kDa heat shock protein, mitochondrial	IPI00339148
488	0.0406	decreased	Dihydrolipoylly sine-residue succinyltrans ferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	IPI00551702 IPI00948493
523	0.0373	depends on experimental group	Pyruvate kinase isozymes M1/M2	IPI00231929
577	0.0241	decreased	Beta-enolase	IPI00231631
			Cytochrome b-c1 complex subunit 1, mitochondrial	IPI00471577

Cont.

Spot number	1w-ANOVA	Relative fluorescence intensity	Protein identification	Protein accession numbers
716	0.0195	decreased	Creatine kinase M-type	IPI00211053
			Aspartate aminotransferase, cytoplasmic	IPI00421513
			Fructose-bisphosphate aldolase A	IPI00231734 IPI00951991
749	0.0188	decreased	Protein S100-A11	IPI00554148 IPI00766347
			Annexin A1	IPI00231615 IPI00777179
935	0.0124	decreased	Malate dehydrogenase, cytoplasmic	IPI00198717
971	0.00166	decreased	Voltage-dependent anion-selective channel protein 2	IPI00198327 IPI00206268
			myozenin-1	IPI00199718
1078	0.00636	increased	Carbonic anhydrase 3	IPI00230788
1173	0.0242	increased	Glutathione S-transferase Yb-3	IPI00230942 IPI00411230 IPI00778425 IPI00870234
			Triosephosphate isomerase	IPI00231767
1196	0.0199	decreased	Pyruvate kinase isozymes M1/M2	IPI00231929 IPI00339197 IPI00454375 IPI00561880 IPI00764193 IPI00777829 IPI00948028 IPI00957976 IPI00968449

Muscle proteome was analyzed, differential protein spots were picked and in-gel digestion was performed. Proteins were identified by mass spectrometry. For each spot: the 1way-ANOVA value, spot relative fluorescence intensity, protein identification, and protein accession numbers are presented.

Twenty-six protein spots with significantly different fluorescence intensities, at least between two of the experimental groups, were identified (1w-ANOVA, $p \leq 0.05$) (Fig. 2.1S). Unequivocal protein identification by mass spectrometry was achieved for seventeen of the selected spots. There were spots containing more than one protein due to co-migration, so a total of twenty-seven proteins were identified. In this study, a maximum of three proteins per spot were found. Two proteins, serum albumin and pyruvate kinase, were present in three spots, leading to the identification of twenty two unique proteins in EAMG (Table 2.1).

As expected, most identified proteins detected by 2D-DIGE were cytoplasmic, probably due to the bias of detecting soluble proteins using this technique. We observed

changes by 2D-DIGE in β-enolase and carbonic anhydrase III (CAIII) (Fig. 2.1*A, B*), and quantified their protein levels in control and EAMG animals by Western blotting. Protein levels of β-enolase were significantly reduced in EAMG muscles compared with controls (n = 6 for both groups, $p < 0.05$), with an average reduction of approximately 25% (Fig. 2.1*C*). CAIII protein levels were increased in all EAMG stages when quantified by immunoblotting (data not shown). Interestingly, only severely affected animals (score 3) had significantly higher levels of CAIII protein compared with controls. In such muscles, average levels of CAIII were increased by approximately 35% compared with control muscles ($p < 0.01$; n = 5 for score 3 EAMG and n = 10 for control) (Fig. 2.1*D*).

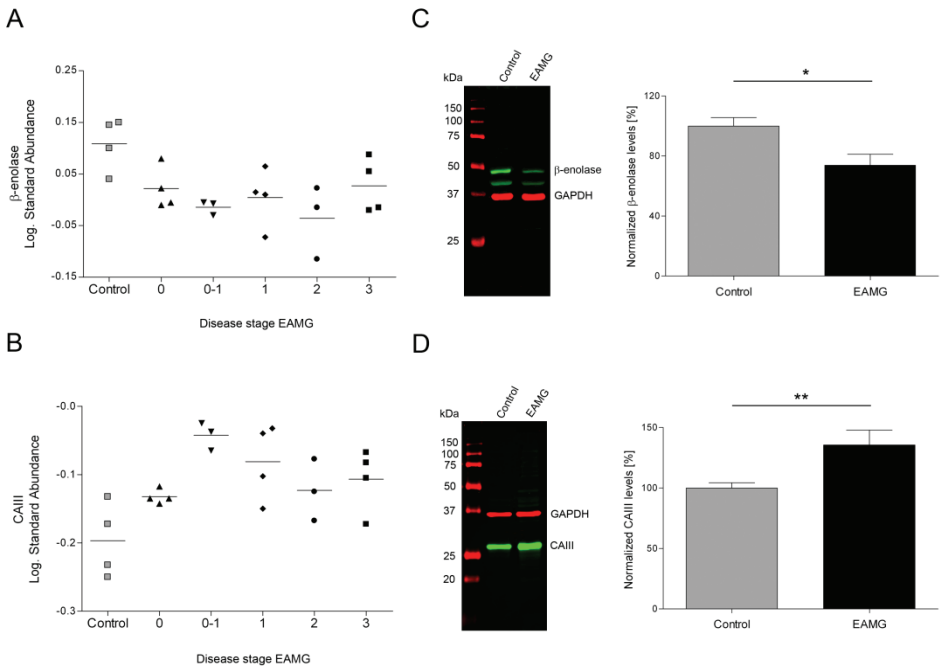

Figure 2.1. β-enolase and CAIII protein levels in tibialis anterior muscles of EAMG and control rats. Both a reduction of β-enolase (A) and an increase of CAIII (B) levels were detected by 2D-DIGE in all EAMG disease stages. Reduction in β-enolase protein levels were corroborated by Western blotting (C). A significant reduction in muscle homogenates from EAMG animals compared with controls (* $p < 0.05$, n = 6 per condition) was observed. CAIII protein levels measured by Western blotting (D) were significantly increased in muscle homogenates from severely affected EAMG animals compared with controls (** $p < 0.01$; score 3 EAMG, n = 5, control n = 10). β-enolase (C upper green band) / CAIII intensity (D green band) were normalized with GAPDH intensity (red band).

Discussion

We analyzed the muscle proteome at different EAMG disease stages by 2D-DIGE and identified twenty-two differentially expressed proteins. The majority of these identified proteins are involved in metabolic pathways (glycolysis and the citric-acid cycle), while others are related to cellular-stress responses (e.g. glutathione S-transferase Yb3, 60 KDa heat shock protein), or are contractile proteins (myosin-4 and myozenin-1). Overall, we observed a reduction of the glycolytic capacity and of fast-twitch myosin isoforms in EAMG tibialis anterior muscles, which suggests a switch from fast- to slow-twitch fibers. Similar proteomic profiles were previously described in conditions that impair muscle contraction both in animals and in human diseases, such as denervation [11, 12], inclusion body myositis [13], immobilization [14] and aging [15]. In such studies, relatively few differentially expressed proteins were identified (between 17 and 73), most of them also related to cellular-stress responses and to changes in the type of muscle fibers. Therefore, it appears that the most evident protein alterations in EAMG are a consequence of the impaired muscle-nerve signaling and, possibly, of the atrophy (or loss) of fast-twitch fibers; as it was previously demonstrated in muscle biopsies from MG patients [16-18]. In this connection it might be relevant that enhancing the response of fast-twitch muscle cells with a selective troponin activator improves muscle strength in EAMG [19].

Most of the proteins found in our study are not specific for MG, since they are also affected in other pathological (and physiological) conditions of the muscle. Nonetheless, β-enolase and CAIII have been associated with muscle regeneration and autoimmunity respectively. β-enolase is a muscle-specific metabolic enzyme very sensible to physiological stimuli [20], with an important role in developing and regenerating muscles [21]. Moreover, its deficiency leads to severe myalgias, muscle weakness and fatigability in affected individuals [22]. CAIII is a muscle-specific enzyme that catalyzes the hydration of carbon dioxide and can protect the cell from oxidative damage [23]. It has been described as an auto-antigen in several autoimmune diseases, e.g. rheumatoid arthritis and systemic lupus erythematosus [24], and its expression is reduced in pectoralis muscle biopses of MG patients [25]. The discrepancy in CAIII expression between our experiment (increased) and MG biopses (decreased) is probably explained by differences

in the experimental design. In this regard, timing seems essential because it has been reported that CAIII levels are reduced immediately after denervation, but they subsequently increase over basal levels six days after the procedure [12].

The NMJ area, with a width of 10 µm, is no more than 1/3000 of the 30 mm total length of the tibialis anterior fiber and, even in such an imaginary transverse 10 µm section, the NMJ proteins would still be vastly outnumbered by the proteins of the muscle. Therefore, it is not unexpected that we could not identify any changes in well-known NMJ-related protein between EAMG and control muscles [26, 27]. Moreover, membrane proteins are considerably less abundant than cytoplasmic proteins and, since they usually have several hydrophobic domains, they are poorly soluble in the aqueous buffers required for isoelectric focusing [28, 29]. Furthermore, there is high inter-individual variation within the EAMG model, which also limits the robustness of 2D-DIGE for detecting differentially expressed proteins, especially those present at low quantities.

At present, the response of muscle fibers to the autoimmune attack in MG remains poorly understood. This study describes alterations in cytoplasmic proteins that are not intuitively considered to be involved in the pathogenesis of MG, and it suggests that AChR-MG shares similar intracellular disease mechanisms with other muscle pathologies.

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Chapter 3

Silencing of Dok-7 in adult rat muscle increases susceptibility to experimental autoimmune myasthenia gravis

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Abstract

Myasthenia gravis (MG) is an autoimmune disease mediated by autoantibodies that target proteins at the neuromuscular junction (NMJ); primarily the acetylcholine receptor (AChR) or the muscle-specific kinase (MuSK). In MG, the muscle responds to an autoimmune attack by up-regulating proteins that can compensate for the loss of function (e.g. AChR, MuSK, rapsyn, Dok-7). Since Dok-7 is essential for the full activation of MuSK and consequently for dense clustering of AChRs, we hypothesized that reduced levels of Dok-7 increases the susceptibility to passive transfer experimental autoimmune myasthenia gravis (EAMG) and impairs recovery after the antibody attack. Dok-7 expression was silenced by transfecting shRNA coding plasmids into the tibialis anterior muscle of adult rats by *in vivo* electroporation. Mild/subclinical MG was subsequently induced with 5 pmol/100 g body weight of anti-AChR monoclonal antibody (mAb) 35. Neuromuscular transmission was significantly impaired in Dok-7-silenced legs when compared with the contralateral control legs. This was correlated with a reduction of AChR levels (~25%) in Dok-7 silenced muscles, as measured by quantitative immunohistochemistry. Furthermore, we examined the effects of Dok-7 silencing in the recovery from passive transfer EAMG (20 pmol mAb35/100 g body weight) by performing electromyography 2, 7, or 14 days after electroporation. Neuromuscular transmission was equally impaired in both Dok-7 silenced and non-silenced legs at all time points studied. These results suggest that a reduced expression of Dok-7 is not fundamental for the recovery of the NMJ after damage but may play a role in the susceptibility to EAMG, by rendering AChR clusters less resistant to the autoantibody attack.

Introduction

The neuromuscular junction (NMJ) is the synaptic connection between a motor nerve terminal and the skeletal muscle membrane. For an efficient neuromuscular transmission to occur, acetylcholine receptors (AChRs) must be densely clustered on the top of postsynaptic-membrane folds facing the nerve terminal, in a process primarily mediated by the activation of muscle-specific kinase (MuSK). To be activated, MuSK requires two signals: one from nerve-derived agrin and one from the muscle cytoplasmic protein downstream of kinase 7 (Dok-7) [1, 2]. Once released from the nerve terminal, agrin binds to its receptor, the low-density lipoprotein receptor-related protein 4 (Lrp4), and this, in association with basal activation by Dok-7, activates MuSK at the muscle's postsynaptic membrane. This ultimately leads to dense AChR clustering by the receptor associated protein (rapsyn). All knock-out models of the main AChR-clustering proteins (rapsyn, MuSK, Dok-7 and Lrp4) die at birth due to an absence of mature NMJs that causes respiratory failure [2-5], demonstrating the crucial role of each of these proteins during embryonic synaptogenesis [2, 6, 7].

The adaptor protein Dok-7 is the most recently described member of the AChR-clustering pathway, and it is localized in the intracellular face of the postsynaptic membrane in close association with MuSK. Recessive mutations in the DOK7 gene are a frequent cause of congenital myasthenic syndromes (CMS) [8, 9]. Patients with such mutations develop a "limb girdle" pattern of muscle weakness at an early age. In muscle biopsies of these patients simplified endplates with reduced postsynaptic folding and impaired neuromuscular transmission are found [10-12]. These observations suggest the possibility that Dok-7 could also have an important role in the pathophysiology (and treatment) of acquired diseases that affect the NMJ in adulthood, such as myasthenia gravis (MG).

In AChR-MG, autoantibodies cause complement-mediated damage at the NMJ, leading to loss of AChR and AChR-associated proteins [13]. Loss of synaptic proteins may impair the clustering of newly-synthesized AChR and delay the NMJ recovery from antibody-mediated damage. Thus, it can contribute to the severity of symptoms in MG.

Silencing the expression of rapsyn in adult muscles with small interfering RNAs (siRNAs) impaired neuromuscular transmission [14], while its over-expression protected the NMJ from antibody-mediated damage in the experimental autoimmune myasthenia gravis model (EAMG) [15]. In addition, adult NMJs were disrupted when MuSK expression was reduced by siRNAs [16], or by its conditional inactivation in transgenic mice [17], which further implies an important role for AChR-associated proteins in the maintenance of the NMJ.

In order to obtain further insight into the role of Dok-7 in adult NMJs, we studied the effect of down-regulation of Dok-7 by RNA interference in control and EAMG muscles. Adult muscles of rats were transfected by *in vivo* electroporation using the mammalian expression vector pSUPER that directs the synthesis of short hairpin RNA (shRNA) transcripts. The results suggest that reduced Dok-7 expression is not crucial for the recovery from the damage caused by autoantibodies, but it increases the susceptibility to EAMG.

Materials and Methods

shRNA constructs and evaluation of silencing efficiency

Dok-7 siRNA sequence selection was performed following Tuschl rules [18, 19], using the siRNA design interface from the University of Hong Kong (http://i.cs.hku.hk/~sirna/software/sirna.php). A total of 5 different siRNAs, targeting nucleotides sequences conserved between rat (NM_001130062) and mouse (NM_172708) Dok-7, plus 1 scrambled siRNA (negative control designed by random mutations in the sequence of Dok-7 siRNA5), were cloned in the pSUPER vector [20] and subsequently verified by sequencing as described [14].

Dok-7 silencing efficiency was measured *in vitro* by co-transfecting the shRNA-pSUPER and scrambled constructs with a rat Dok7–GFP expression vector (rat Dok-7, NM_001130062 in a pEGFP-N1 vector, cloned by Geneart AG, Regensburg, Germany) in HEK 293 cells. HEK cells were cultured in DMEM medium with 10% heat-inactivated fetal bovine serum (iFBS) and transfected with 2.5 µg of each plasmid using the

polyethylenimine (PEI) transfection method [21], in the presence of D-glucose (6.5% w/v). Dok-7 silencing efficiency in transfected cells was estimated by measuring GFP intensity with an Olympus IX-81 inverted fluorescence microscope (Olympus Nederland B.V., Zoeterwoude, the Netherlands), and by quantification of Dok-7 protein levels in cell extracts by Western blotting. Dok-7 intensity levels were quantified using ImageJ software (http://rsb.info.nih.gov/ij/); each condition was analyzed in triplo.

C2C12 myoblast cells were co-transfected with Dok-7 shRNAs and Dok7-GFP using a NEON transfection system (Invitrogen, Paisley, UK), and were cultured in DMEM medium plus 15% iFBS until confluence. Cells were then switched to DMEM medium plus 5% iFBS for differentiation and, once myotubes were formed, AChR clusters were stained with alpha-bungarotoxin Alexa 594 (B-13423, Invitrogen, Paisley, UK; 1:1000 diluted), as described [9]. A total surface of 4.25 mm^2 was imaged per condition (from 3 replicates) with an Olympus IX71 fluorescence microscope (Olympus UK, Southend-on-Sea, UK) and the average AChR cluster length was estimated with ImageJ.

Based on their *in vitro* silencing efficiency, we selected 2 shRNAs, in addition to the scrambled shRNA, for muscle electroporation *in vivo* (Table 3.1).

Table 3.1. Primer sequences for silencing Dok-7 expression using pSUPER plasmid.

shRNA1 (S-1) fwd	5'-GATCCCCGCAGATCAGCTTCCTGTTTttcaagagaAAACAGGAAGCTGATCTGCTTTTTGGAAA-3'
shRNA1 (S-1) rev	5'-AGCTTTTCCAAAAAGCAGATCAGCTTCCTGTTTtctcttgaaAAACAGGAAGCTGATCTGCGGG-3'
shRNA5 (S-5) fwd	5'-GATCCCCGATCAGCTTCCTGTTTGACttcaagagaGTCAAACAGGAAGCTGATCTTTTTGGAAA-3'
shRNA5 (S-5) rev	5'-AGCTTTTCCAAAAAGATCAGCTTCCTGTTTGACtctcttgaaGTCAAACAGGAAGCTGATCGGG-3'
Scrambled shRNA fwd	5'-GATCCCCGAGATGGTCCCTGTTTGACAGttcaagagaCTGTCAAACAGGGACCATCTCTTTTTA-3'
Scrambled shRNA rev	5'-AGCTTAAAAAGAGATGGTCCCTGTTTGACAGtctcttgaaCTGTCAAACAGGGACCATCTCGGG-3'

Animals and tissue processing

7-week-old female Lewis rats were obtained from Charles River laboratories (Cologne, Germany), with permission from the Committee on Animal Welfare, according to Dutch governmental rules. For electroporation, induction of passive-transfer EAMG and electromyography, animals were anesthetized and later euthanized as described [14]. Frozen muscles were cut in a cryostat (CM3050 S, Leica Biosystems, Wetzlar, Germany) in 10 µm sections (for immunohistochemistry and immunofluorescence) and 50 µm sections (for protein and RNA extraction). For electron microscopy analysis, animals were

euthanized as previously described [15, 22] with the following modifications: fixation buffer was 3% glutaraldehyde plus 1.4% sucrose buffered in 0.09 M KH_2PO_4, pH 7.4; a mix of ketamine (100 mg/kg)/xylazine (15 mg/kg) was used for anesthesia; and fixed muscles were sectioned at 500 µm with a VT1200S vibratome (Leica Biosystems, Wetzlar, Germany).

Electroporation and optimization of Dok-7 silencing in vivo

Dok-7-shRNAs plasmids were prepared with the Qiagen Gigaprep DNA purification kit (cat. 12191), according to manufacturer's manual, and dissolved at a concentration of 2 µg/µl in 0.9% NaCl. Electroporation of the tibialis anterior muscle was performed with the same parameters as described previously [14, 15], using the Electro Square Porator ECM 830 (BTX, San Diego, USA). Dok-7-silencing was performed by injecting muscles with 50 µg of each of the two silencing vectors (S-1 and S-5, see table 3.1) and, for the contralateral control leg, 100 µg of the scrambled shRNA vector in a final volume of 50 µl. To estimate the transfection and silencing efficiency, a group of 18 rats were injected with a DNA solution containing 100 µg of S-1 or S-5, plus 50 µg of pVAX1-LacZ in a volume of 75 µl. These rats were later sacrificed at 7, 14 or 28 days after electroporation (3 animals per time point). The reporter gene ß-galactosidase was highly expressed 7 days after electroporation in approximately 60% of the muscle fibers and it was still detectable at day 28 after electroporation (Fig. 3.1*A*). Dok-7 protein levels were 16% reduced in Dok-7 silenced legs compared with non-silenced legs 28 days after electroporation. Importantly, this reduction was not observed at early time points (supplementary Fig. 3.1), indicating that the *in vivo* down-regulation of Dok-7 with shRNAs is likely to require, at least, 14 days to manifest, as previously described also for down-regulation of MuSK [16].

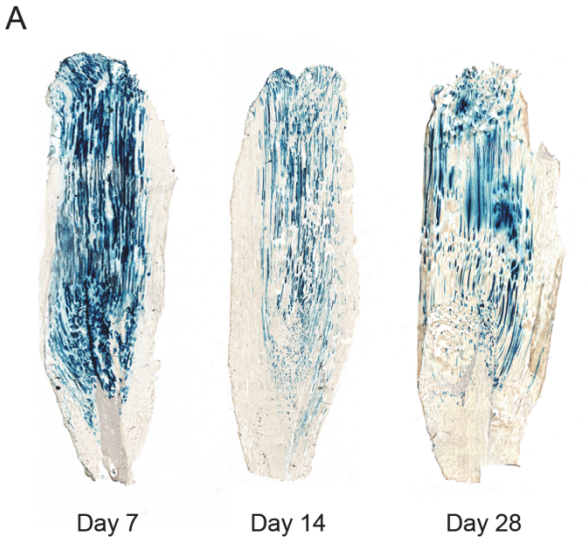


Figure 3.1. (A) In vivo electroporation of shRNA plasmids. The transfection efficiency was analyzed using X-gal as a substrate for the reporter β-galactosidase. The sections are spaced 500 µm. ß-galactosidase expression was observed in muscle fibers at all analyzed time points (7, 14 and 28 days after electroporation), indicating stable plasmid expression. Scheme of the experimental set up for investigating the effects of in vivo Dok-7 silencing in (B) the susceptibility to EAMG, and (C) the recovery from autoantibody damage in EAMG.

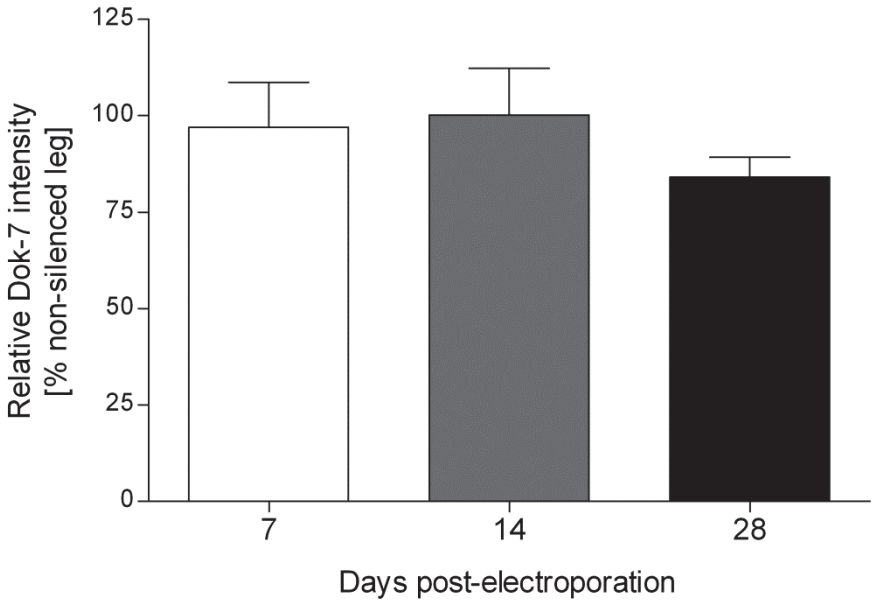


Figure 3.1S. In vivo silencing efficiency of Dok-7 shRNAs by muscle electroporation. Dok-7 levels were analyzed by Western blotting in muscle protein extracts from animals electroporated with Dok-7 shRNAs at 7, 14 or 28 days after electroporation (n=3 per time point). Relative Dok-7 and GAPDH intensities were quantified by ImageJ and results are expressed as the percentage of Dok-7/GAPDH ratio in the Dok-7 silenced leg compared with the contralateral non-silenced leg. Dok-7 protein levels were not reduced at 7 or 14 days after electroporation, but they were decreased by 16% in Dok-7 silenced legs, compared with the contralateral no-silenced leg, at day 28.

Induction of EAMG and experimental set-up

The effects of Dok-7 silencing at the NMJ were subsequently investigated in EAMG animals following two different experimental set-ups. In both cases, animals were electroporated on day 0 and, on day 35, EAMG was induced by intraperitoneal injection of anti-AChR monoclonal antibody (mAb) 35 [23], while control animals were untreated. One EAMG group received 5 pmol/100 g body weight (bw) mAb35 (subclinical dose, see supplementary Fig. 3.2) and was sacrificed 48 h later (*n*=5 EAMG and *n*=3 control rats) in order to evaluate the importance of Dok-7 for the susceptibility to EAMG (Fig. 3.1*B*). The other EAMG group received 20 pmol/100 g bw mAb35 (standard dose) and was sacrificed at 2, 7 or 14 days after induction of EAMG (*n*=5 EAMG and *n*=3 control rats per time point) for assessing the relevance of Dok-7 in the recovery from the damage induced by

autoantibodies (Fig. 3.1*C*). This standard dose of mAb35 leads to acute muscle weakness and severely impaired neuromuscular transmission [24], which normally fully recover within 1 week after induction of passive-transfer EAMG [25, 26]. Clinical scoring was evaluated daily with the paw-grip test as previously described [15, 22].

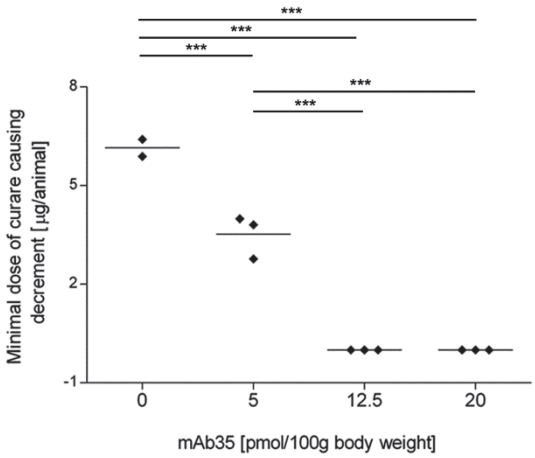


Figure 3.2S. Assessment of the minimal curare dose that induced decrement (subclinical EAMG). Rats were injected with 5, 12.5 or 20 pmol/100 g bw mAb35 (n=3 per dose), and neuromuscular transmission was assessed by EMG 2 days later, in the presence of continuous i.p. injections of curare. Animals that received either 12.5 or 20 pmol/100 g bw mAb35 had a decrement already before the injection of curare solution, while those injected with 5 pmol/100 g bw required approximately 3.5 µg curare to show a decremental response. This was significantly less curare than what was needed to induce decrement in control animals (5 µg), indicating that injection of 5 pmol/100 g bw mAb35 significantly impaired neuromuscular transmission, though to a lesser extent than injection of 12.5 or 20 pmol/100 g bw mAb35. Additionally, at 2 days after induction of EAMG, animals that received 12.5 or 20 pmol/100 g bw mAb35 had clinical scores ranging from 0 to 2, while control animals and animals injected with 5 pmol/100 g bw mAb35 had no clinical signs of muscle weakness (score 0, data not shown). *** $p < 0.001$; 1-way ANOVA.

Electromyography (EMG)

Decrement of compound muscle action potential (CMAP) was measured simultaneously in both tibialis anterior muscles of *n*=5 EAMG and *n*=3 control rats (per time point) at the day of sacrifice, as described [14, 15]. If no decrement was observed initially, neuromuscular transmission was challenged by a continuous *i.p.* infusion of a 20 µg/ml solution of curare

[(+)-tubocurarine, T2379; Sigma-Aldrich], at a rate of 1ml/h (0.33 µg curare/min) with a syringe pump (SP210CZ, World Precision Instruments Germany GmbH, Berlin). During curare infusion, CAMP measurements were repeated at intervals of 1 min (for each leg), until a reproducible decrement was observed. All EMG studies were performed by the same investigators.

Immunohistochemistry

Muscle cryosections of 10 µm from pVAX-ß-galactosidase transfected animals were stained for ß-galactosidase activity as described [14], dehydrated in a series of ethanol dilutions (70–100%), immersed in Ultraclear (J.T Baker, the Netherlands), and coverslipped with Pertex (Histolab Products AB, Göteborg, Sweden). Sections were imaged with an Olympus BX50 microscope (Tokyo, Japan) and the Stereo Investigator software (Microbrightfield Bioscience, VT).

To visualize NMJs, 10 µm frozen muscle sections were fluorescently stained. Cryosections were fixed in cold acetone for 15 min at 4°C and, subsequently, muscle slides were delimited with a PAP hydrophobic pen. Sections were blocked for 30 min with donkey serum (1:500 in TBS-T), while endogenous biotin-binding sites in the muscle were blocked with a streptavidin/biotin blocking kit (SP-2002, Vector Laboratories, Burlingame, CA), following the manufacturer's instructions. Slides were then incubated for 1 h with the following primary antibodies: rabbit anti-MuSK (1:1,000; kind gift from Steven J. Burden, New York University, NY) and mouse anti-SV2 (directed against synaptic vesicles; 1:2,000; DS Hybridoma Bank, IA). Alexa-647 conjugated alpha-bungarotoxin (1:300; B35450, Invitrogen/Molecular Probes, Bleiswijk, the Netherlands) was utilized for detection of AChRs. Subsequently, slides were incubated 1 h with the following secondary antibodies: biotin-conjugated goat anti-rabbit IgG (1:1,000; 111-065-144, Jackson ImmunoResearch, West Grove, PA) and Alexa 594 donkey anti-mouse IgG (1:300; A21203, Invitrogen/Molecular Probes), followed by incubation with Alexa 488 streptavidin (1:2,000; S11223 Invitrogen/Molecular Probes) for another hour at RT. Finally, slides were mounted in 80% glycerol-TBS. All antibodies and reagents were diluted in TBSA (2% bovine serum albumin in TBS) and all washing steps were performed with TBS-T.

Quantitative immunofluorescence analysis

The relative concentrations of AChR and MuSK at the NMJ were analyzed as previously described [14, 15]. Muscle sections from *n*=5 EAMG and *n*=3 control rats (16 muscles in total) were stained for visualization of AChR, MuSK and SV2 as described above. For each muscle, 2 sets of sections from 2 different regions of the tissue were stained, each set containing 3 muscle sections of 10 µm. Endplate regions were identified based on their morphology and positive staining for SV2, and they were imaged for the quantification of SV2, AChR and MuSK intensities with an Olympus Provis AX70 fluorescence microscope (Olympus Nederland B.V.). Approximately 50 endplate regions per muscle (with 3-10 endplates each) were imaged and the fluorescence intensities were quantified using ImageJ. For each single endplate, both AChR and MuSK mean intensities were normalized to the SV2 mean intensity, to allow comparisons between different postsynaptic regions. The average of the normalized intensities from all endplates in a set of muscle sections was used for comparison between conditions.

Electron microscopy

Electron micrographs were obtained from endplate regions of the tibialis anterior muscles of *n*=5 EAMG rats and *n*=3 control rats with unilateral Dok-7 silencing. Sections of glutaraldehyde-perfused muscles (500 µm) were processed for EM imaging as described [15, 22]. Ultra-thin sections (80 nm) were viewed with a Philips CM 100 electron microscope. At least 15 endplate regions (including between 1 to 3 endplates) were imaged from each muscle and the morphometric analysis of each micrograph was performed using the ImageJ software, as described [15, 22, 27, 28].

Protein extraction and immunoblotting

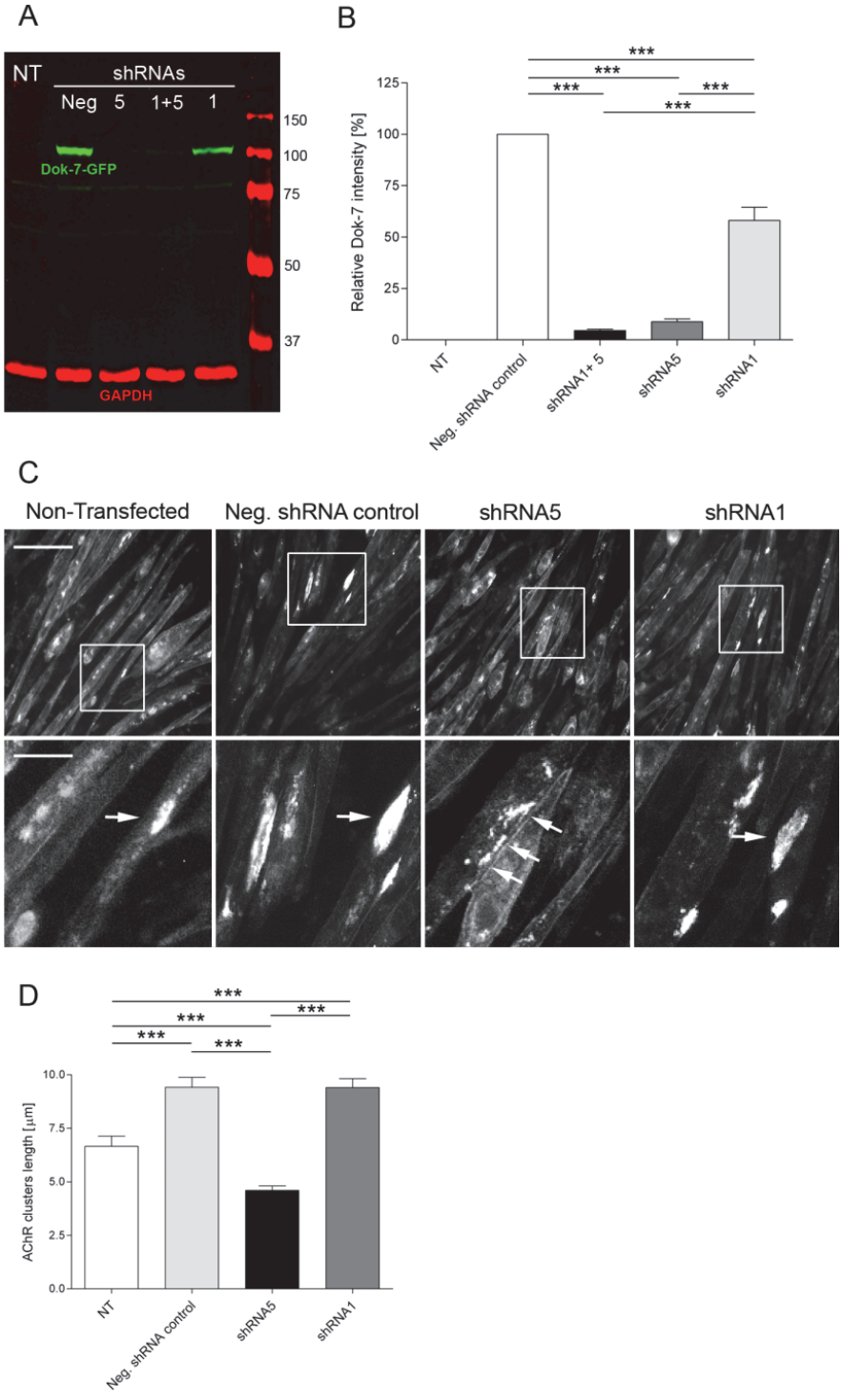
Muscle sections (50 µm) were homogenized in approximately 5 mL of protein extraction buffer A (10 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L NaN3, 10 mmol/L iodoacetamide, and 1 mmol/L phenylmethyl sulfonyl fluoride in PBS) by vortexing 3 times for 1 min (with 1 min intervals), in the presence of glass beads (0.5 mm). The extraction buffer also contained protease inhibitors (Complete Cocktail Tablets, Roche Diagnostics,

the Netherlands) and phosphatase inhibitors (PhosSTOP, Roche Diagnostics, the Netherlands). Next, the homogenate was centrifuged at 22,000 g for 30 min and the supernatant was recovered as a cytoplasmic-protein enriched sample. The resulting pellet was resuspended in 1.8 ml of buffer B (buffer A with an additional 2% Triton X-100) for extracting membrane-bound proteins. Extraction was performed for 1 h at 4°C on a reciprocal shaker followed by centrifugation (22,000 g, 30 min) and collection of the supernatant (membrane-enriched protein sample).

Protein extracts from tibialis anterior muscles and from transfected HEK cells were separated by SDS 10% polyacrylamide gel electrophoresis (SDS-PAGE) for 1 h at 160-200V, transferred to a nitrocellulose membrane (Bio-Rad Laboratories, CA) for 1 h at 100V and blocked with 1:2 PBS-diluted blocking buffer (Odyssey blocking buffer, LI-COR Biosciences, NE) for 1 h at room temperature. Membranes were then incubated with either rabbit anti-Dok-7 (H-77, Santa Cruz Biotechnology, CA; 1:400) or rabbit anti-MuSK sera (kind gift from Judy Cossins, Oxford, UK; 1:20,000), and mouse anti-GAPDH (10R-G109a, Fitzgerald, MA; 1:3,000,000), overnight at 4°C. Secondary antibodies were donkey anti-mouse IgG IRDye 680 and goat anti-rabbit IgG IRDye 800 (926-32222 and 926-32211, respectively; LI-COR Biosciences, NE), diluted 1:10,000. Finally, the membranes were imaged with the Odyssey Infrared Imaging System (LI-COR Biosciences, NE) and bands were quantified with the ImageJ software (mean intensity), normalizing for GAPDH expression.

Statistics

GraphPad Prism 4 was used to perform statistical analyses. Comparison between normally distributed values was performed using either a 1-way or 2-way ANOVA, and Bonferroni post hoc test. A two-sided probability value (p) <0.05 was considered significant. Values are expressed as mean ± standard error of the mean (SEM), unless stated otherwise.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NT
Neg. shRNA control
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Figure 3.2. In vitro analysis of shRNA efficiency. (A) HEK 293 cells were co-transfected with plasmids for the expression of Dok-7-GFP (MW= 91 kDa), and anti-Dok-7 shRNAs (S-1, S-5 and S-1 + S-5) or a scrambled shRNA expression plasmid pSUPER. 48 h later, cells were harvested and protein extracts were separated by SDS-PAGE, followed by immunoblotting for Dok-7 (green band) and GAPDH (red band). (B) Western blotting quantification of Dok-7 silencing efficiency using ImageJ densitometry software. Co-transfection of Dok-7–GFP with anti- Dok-7 shRNAs (S-1+ S-5 and S-5) strongly reduced Dok-7 expression compared to cells transfected with the scrambled shRNA pSUPER plasmid. Dok-7 band intensities were normalized for GAPDH intensity and results are expressed as the percentage of Dok-7 intensity compared with the negative control shRNA (scrambled). Error bars indicate the standard error of the means of 3 independent experiments. (C) In vitro analysis of Dok-7 shRNA efficiency in C2C12 cells. C2C12 myoblasts were co-transfected with plasmids for the expression of Dok-7-GFP, and anti-Dok-7 shRNAs (S-1 and S-5) or the scrambled shRNA expression plasmid pSUPER. Upon myotube differentiation, Dok-7-GFP transfected cells had large AChR clusters when no Dok-7 shRNA was co-transfected (see neg. shRNA control). AChR clusters in S-5 co-transfected myotubes were small and disperse, and even smaller than in non-transfected cells. Squares in the upper row delimitate the region enlarged in the lower row, arrows in the lower row point at AChR clusters (D) The average AChR cluster length was increased after co-transfection of Dok-7-GFP with the scrambled shRNA, but it was significantly reduced when Dok-7-GFP was co-transfected with shRNA S-5. ImageJ densitometry software was used for quantification of clusters length. *** $p < 0.001$; 1-way ANOVA.

Results

Dok-7 shRNAs reduced Dok-7 levels and induced smaller and simplified AChR clusters in vitro

In order to knock-down the expression of Dok-7 *in vitro* and *in vivo* in adult rat muscles, we designed five shRNA oligonucleotides (S-1–S-5) targeting Dok-7 which were cloned into the pSUPER expression vector and initially tested *in vitro* by co-transfection with Dok-7–GFP in HEK 293 cells. Following transfection, two pSUPER constructs (S-1 and S-5, table 3.1) efficiently reduced the levels of Dok-7–GFP (between 40% and 90%) compared with Dok-7–GFP co-transfected with the scrambled pSUPER vector, as measured by Western blotting ($p < 0.001$, 1w-ANOVA) (Fig. 3.2*A, B*). The combination of these 2 shRNAs had additive effects and showed a reduction of Dok-7–GFP levels of 95% ($p < 0.001$, 1w-ANOVA) (Fig. 3.2*A, B*). Similar Dok-7 silencing efficiencies were measured by fluorescence activated cell sorting (FACS) and by fluorescence microscopy in separate experiments (data not shown).

Since over-expression of Dok-7 leads to increased AChR clustering in muscle cells [2], we tested if shRNAs S-1 and S-5 affected clustering of AChRs in differentiated myotubes of the mouse muscle cell line C2C12. Co-transfection of Dok-7 with S-5 impaired the Dok-7 induced clustering of AChRs in C2C12 cells and led to smaller and simplified AChR clusters (51% smaller than in neg. control shRNA transfected cells, $p < 0.001$; Fig.

3.2*C, D*). In contrast co-transfection of Dok-7 with the scrambled shRNA or S-1 promoted the formation of larger AChR clusters (41% larger than in non-transfected cells, $p < 0.001$). Interestingly, clusters in S-5-transfected C2C12 cells were even smaller than those in Dok-7 non-transfected cells (31%, $p < 0.001$; Fig. 3.2*C, D*).

Effects of Dok-7 silencing in neuromuscular transmission

In order to evaluate the importance of Dok-7 for the susceptibility to EAMG, we reduced levels of Dok-7 in the tibialis anterior muscle by electroporation, subsequently induced EAMG in some animals by injection of 5 pmol/100 g bw mAb35 (subclinical dose), and sacrificed them 2 days later. Silencing of Dok-7 in tibialis anterior muscles of control animals did not impair neuromuscular transmission, as measured by the resistance to curare with EMG (*n*=3; Fig. 3.3*A*). However, Dok-7 silenced muscles in subclinical EAMG animals were less resistant to curare (26% less), when compared with the non-silenced contralateral leg (*n*=5); $p < 0.05$; Fig. 3.3*A*).

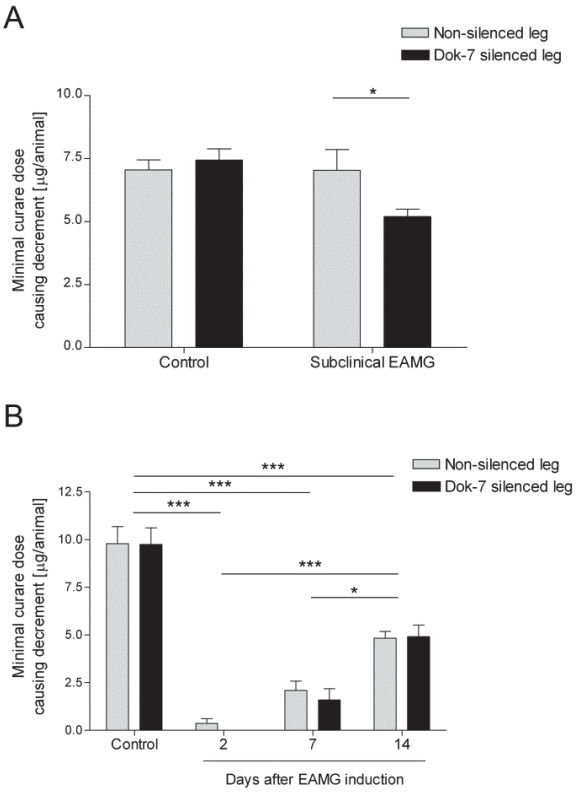

Figure 3.3. Silencing of Dok-7 impairs neuromuscular transmission in subclinical EAMG (induced with 5 pmol/100 g bw). CMAP was measured bilaterally in transfected muscles by EMG, in the presence of continuous curare i.p infusion. (A) Silencing of Dok-7 did not affect the resistance to curare of control animals, but it significantly decreased curare resistance in subclinical EAMG animals. Control n=3, EAMG n=5. (B) Dok-7 silencing had no significant effect on the recovery from autoantibody damage up to 14 days after injection of 20 pmol/100 g bw of mAb35. At this time point, curare resistance was still significantly lower than in control animals, indicating that neuromuscular transmission was not fully recovered yet. Control n=3, EAMG n=5 per time point.*** $p < 0.001$, * $p < 0.05$; 2-way ANOVA.

For evaluating the effects of Dok-7 silencing in the recovery from EAMG, we measured neuromuscular transmission at 2, 7 and 14 days after injection of mAb35 to induce a full-blown disease (20 pmol/100 g bw). As anticipated from the abovementioned experiment, no differences were observed in the curare resistance of Dok-7 silenced and non-silenced legs in control animals (*n*=3). Unexpectedly, this was also the case for EAMG animals in all the time points evaluated. There were no significant differences in the resistance to curare between Dok-7 silenced and non-silenced legs at 2, 7, or 14 days after induction of EAMG (*n*=5 per time point; Fig. 3.3*B*). A tendency for less curare resistance in the Dok-7 silenced leg was observed at 2 and 7 days time points, although it was not statistically significant. This suggests that a lower expression of Dok-7 at the NMJ is possibly not crucial for the recovery from the damage caused by autoantibodies. However, it is important to note that, 14 days after the induction of EAMG, both Dok-7 silenced and non-silenced legs had a lower curare resistance than that of control animals, suggesting that NMJs were not totally regenerated after the structural damage.

Dok-7 silencing reduces AChR protein levels in EAMG rat muscles

To further characterize the effects of Dok-7 silencing in AChR and AChR-associated proteins, we stained for AChR, MuSK and SV2 in muscles of rats injected with 5 pmol/100 g bw mAb35 (*n*=5) and in untreated animals (*n*=3), and estimated their relative abundance at the NMJ by quantitative immunofluorescence. The majority of endplates in subclinical EAMG animals had a normal morphology in both Dok-7 silenced and non-silenced legs (Fig. 3.4*A*). The intensities of AChR and MuSK were quantified in approximately 300 endplates per muscle and normalized for their corresponding SV2 intensity, because this presynaptic marker is typically unaffected by EAMG [14]. In control animals, both AChR/SV2 and MuSK/SV2 ratios were not changed by Dok-7 silencing, and they were not significantly different from those in subclinical EAMG (Fig. 3.4*B, C*). By contrast, Dok-7 silenced legs in subclinical EAMG animals had a significantly lower AChR/SV2 ratio at their NMJs compared with non-silenced legs (25% less; $p < 0.01$, Fig. 3.4*B*), while MuSK levels were not significantly affected by Dok-7 down-regulation (Fig. 3.4*C*). These findings are in line with the impaired neuromuscular transmission observed by EMG in Dok-7 silenced

legs of subclinical EAMG animals, but not controls. Altogether, they suggest that functional AChR molecules are more likely to be reduced by the relatively low amount of autoantibodies in subclinical EAMG when there is less Dok-7 available in the muscle.

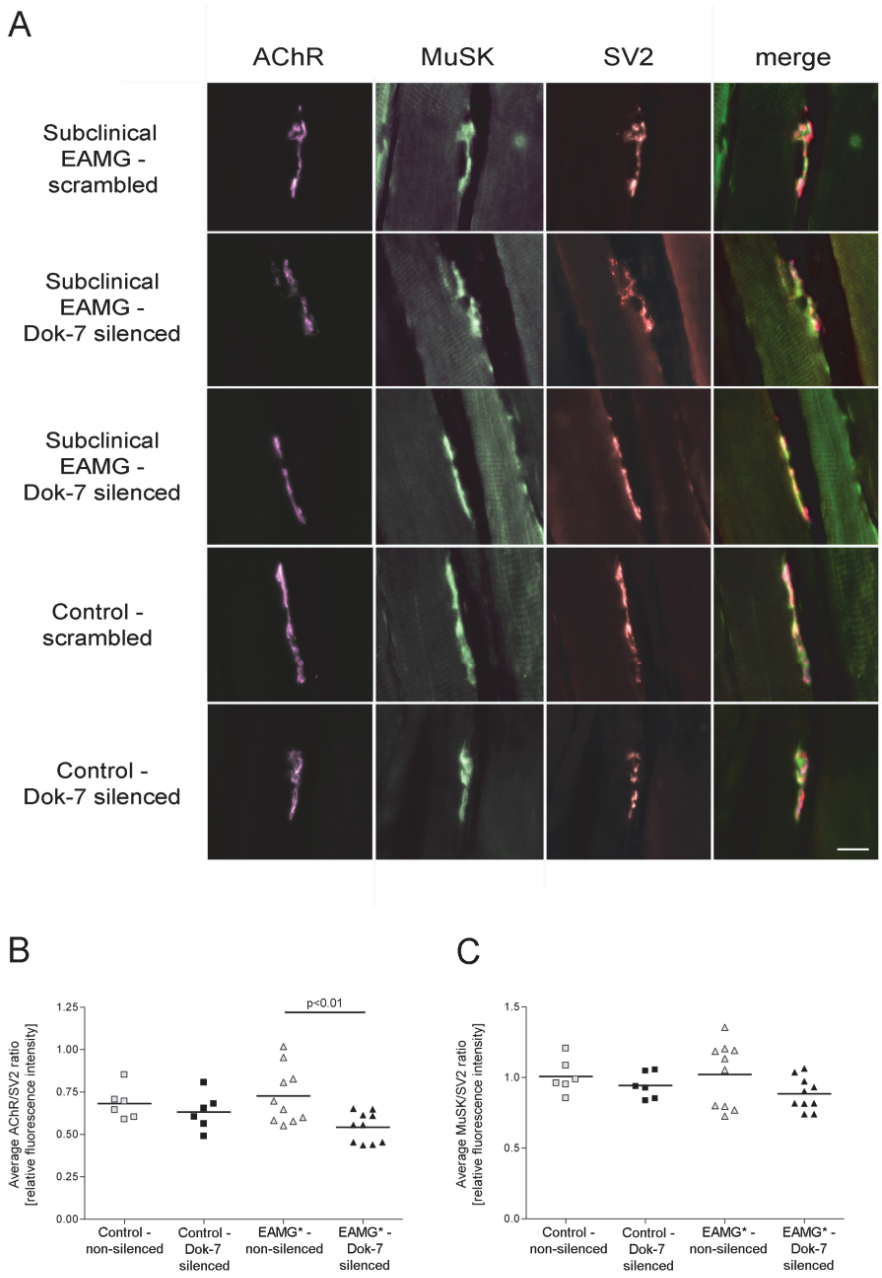

Figure 3.4. Fluorescent microscopy images of representative endplates and quantitative immunohistochemistry analysis. Muscle sections were triple stained for AChR (magenta), MuSK (green) and the presynaptic protein SV2 (red) as a reference. (A) Representative images of endplates from control and subclinical EAMG muscles. No evident differences in morphology or AChR clusters size were observed between Dok-7 silenced and non-silenced legs in control animals. The majority of endplates were normal in subclinical EAMG muscles, though in Dok-7 silenced legs some endplates were disrupted and had less AChR intensity (as representatively shown in the panel). Scale bar is 10 µm. Quantification of the relative intensity of AChR (B) and MuSK (C), normalized for SV2 intensity. The average AChR/SV2 ratio was significantly lower in Dok-7 silenced legs, compared with the contralateral non-silenced legs, in subclinical EAMG animals but not in controls. MuSK levels were not significantly affected by Dok-7 silencing. Approximately 300 endplates per muscle were quantified from 5 EAMG animals and 3 controls. 2-way ANOVA was used for comparisons.

Effects of Dok-7 silencing in NMJ ultrastructure

In addition, we examined the ultrastructure of the NMJ by electron microscopy. Control animals had morphologically normal NMJs in both Dok-7 silenced and non-silenced legs (Fig. 3.5*A, B*), and no particular endplate abnormalities were observed due to Dok-7 silencing. Resembling control animals, most endplates in subclinical EAMG rats also had a normal morphology in both Dok-7 silenced and non-silenced legs. However, some endplates were clearly disrupted at the postsynaptic membrane, with wider junctional folds and enlarged synaptic clefts or simplified postsynaptic foldings, which are usually signs of complement-mediated damage in EAMG (Fig. 3.5*C, D*). Such endplates were equally observed in both Dok-7 silenced and non-silenced legs. For statistical comparisons between conditions, we performed quantitative morphometric analysis of the folding index (ratio between the post- and presynaptic membrane length [29]) in control and subclinical EAMG animals (n=3 and n=5, respectively; approximately 25 endplates per muscle were analyzed). The folding index was not significantly different either between these two conditions (averages per group: control=7; EAMG=6.1) or between Dok-7 silenced and non-silenced legs (averages per group: EAMG non-silenced=6.1; EAMG Dok-7 silenced=5.5).

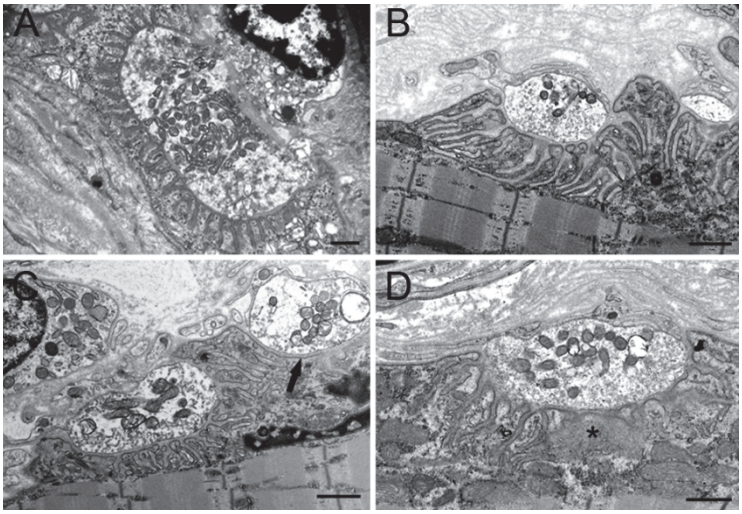

Figure 3.5. Electron microscopic analysis of endplate morphology. (A) Normal endplate region in a non-silenced control muscle, with an elaborate postsynaptic folding facing the nerve terminal. (B) Normal endplate region in a Dok-7 silenced control muscle, no particular morphological alterations were observed in the majority of Dok-7 silenced endplates. (C) Muscles injected with 5 pmol/100 g anti-AChR antibodies (subclinical EAMG) contained both normal endplate regions and endplates showing reduction of postsynaptic folding (see arrow) and widening of the synaptic cleft [see asterisk in (D)]. Ultra-thin muscle sections from 5 EAMG and 3 control animals were imaged with an electron microscope (at least 15 endplate regions per muscle). Scale bars are 1 µm.

Discussion

In this study, we have examined the effects of silencing Dok-7 in order to obtain further insight into the function of Dok-7 in healthy and EAMG adult muscles. Down-regulation of Dok-7 led to an increased susceptibility to mAb35. Unexpectedly, it did not impair the recovery from autoantibody damage up to 14 days after the induction of EAMG. Additionally, silencing of Dok-7 did not affect neuromuscular transmission or led to loss of AChRs in control animals, which suggests that down-regulation of Dok-7 in adult muscle does not affect *per se* the functionality of the NMJ, but it does renders AChR clusters less resistant to autoantibodies in EAMG.

The efficiency of neuromuscular transmission is directly related to the number and degree of clustering of AChRs at the surface of the postsynaptic membrane. The high density of AChR at the NMJ ensures that every nerve stimuli will elicit muscle action potentials, what is also called “the safety factor” of neuromuscular transmission [13, 30].

Since in rodents the safety factor is several folds larger than the minimum threshold needed to induce an action potential (about 5 folds) [31], it is more likely that the effects of reduced Dok-7 expression are only evident when neuromuscular transmission in challenged by an autoimmune attack or a neuromuscular toxin. Indeed, in our experiment we observed a significant impairment in neuromuscular transmission due to Dok-7 silencing only in the presence of autoantibodies, and not in control animals. This impairment, however, was detectable in subclinical EAMG animals but not in rats that received the standard mAb35 dose, most likely because the latter model causes a very extensive damage at the NMJ. Therefore, when analyzed by EMG, standard EAMG animals had typically a decrement in their CMAP without the need for curare injections which, in turn, prevented the detection of small differences in curare sensitivity between Dok-7 silenced and non-silenced legs. In this regard, the mild impairment of neuromuscular transmission in subclinical EAMG animals allowed us to detect these subtle, but significant, differences in the resistance to AChR loss. Moreover, our EMG findings are in good correlation with the quantification of AChRs at the endplate by immunohistochemistry. We observed a significant reduction in AChR content in Dok-7 silenced legs of subclinical EAMG animals, further supporting the importance of Dok-7 in the stabilization of AChR clusters at the NMJ. In this regard, previous studies have demonstrated that Dok-7 is particularly relevant for the pre-patterning of AChR clusters during development [6, 32] and Dok-7 knock-down zebrafish generated fewer and smaller AChR clusters [32]. Additionally, our ultrastructural analysis by electron microscopy did not reveal morphological abnormalities at the endplates of Dok-7 silenced muscles. Thus, it seems that Dok-7 does not play a crucial role in maintaining the ultrastructure of the mature NMJ in the short-term (unlike when rapsyn is silenced, which resulted in increased folding of the postsynaptic membrane [14]) and morphological alterations are probably not responsible for the increased sensitivity to curare of Dok-7 silenced legs.

Dok-7 is a crucial member of the MuSK signaling pathway. Its binding to the juxtamembrane region of MuSK allows for trans-autophosphorylation of the kinase activation loops of MuSK [1], thereby acting as the muscle factor that ensures basal activation of this kinase for full responsiveness to agrin signaling [33]. During embryonic

development, the agrin-MuSK-Dok-7 triad is widely recognized as the main organizer of neuromuscular synapses [2, 6, 34, 35] and, in adulthood, is fundamental for the maintenance and stability of AChR clusters at the NMJ. The latter has been demonstrated by both siRNA down-regulation [16] and conditional inactivation [17] of MuSK expression and, more extensively, by studies that investigated the effects of anti-MuSK antibodies at the NMJ [36-39]. In such studies, the most consistently reported finding was a significant reduction of AChR clustering that follows the decrease in MuSK levels at the endplate; in addition to increased nerve branching, disassembly of endplates, and formation of aberrant NMJs. Although a link between Dok-7 and MuSK expression has been demonstrated by several studies (both proteins share the transcription factor Sp1 [40], Dok-7 levels are the limiting factor for the formation of ectopic AChR clusters when MuSK is overexpressed in muscle fibers [41], and the two proteins increase their expression upon denervation [41, 42]), we did not find significantly changed MuSK levels at the NMJ because of Dok-7 silencing. Given that Dok-7 is required for activation of MuSK and, consequently, for dense and stable NMJs, it seems likely that the loss of AChR clusters by autoantibodies in Dok-7 silenced legs was a result of reduced MuSK activation rather than of reduced MuSK levels.

Induction of EAMG with 20 pmol/100 g body weight is known to cause extensive damage at the NMJ [24], and the clinical and electrophysiological features of passive-transfer EAMG typically recover in about 7 days [25, 26]. Given the previously discussed key role of Dok-7 in neuromuscular synaptogenesis, and its requirement for full activation of MuSK [6], we hypothesized that Dok-7 silencing at the adult NMJ could impair endplate recovery after passive-transfer EAMG. Silencing of Dok-7 in this scenario, however, did not result in a delayed recovery of neuromuscular transmission in any of the time points studied (2, 7, and 14 days), as measured by EMG; even though a trend for an impaired neuromuscular transmission in Dok7-silenced legs was observed. One possibility is that the damage at the NMJ caused by 20 pmol/100 g of mAb35 is so extensive that it overshadows the relatively mild effect of Dok-7 silencing. This is supported by the fact that, even 14 days after mAb35 injection, and in contrast to what was expected, neuromuscular transmission was still not fully recovered in any of the animals'

electroporated muscles. In this regard, it was reported that endplates in passive-transfer EAMG animals require at least 10 days (and possibly up to 54) to fully recover their normal morphology and junctional AChR content after a single injection with autoantibodies [28]. Had we studied the recovery of neuromuscular transmission at later time points (e.g. 21 or 28 days), we might have had a better chance of effectively assessing the effects of Dok-7 silencing on NMJ recovery.

In summary, our results indicate a relevant role for Dok-7 in the susceptibility to damage at the NMJ and, therefore, implicate this protein in the maintenance of a functional NMJ. Taking into account that several well-characterized mutations of the DOK7 gene in humans have been involved in the development of CMS [9-11], it is tempting to speculate that, in the general population, minor variations in the sequence of this gene, its promoter, and the upstream regulators, could also mildly impair the levels or activity of Dok-7. Such carriers would then be more susceptible to damage at the NMJ and, therefore, Dok-7 could be a promising marker to predict the severity of conditions that challenge neuromuscular transmission, such as MG.

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Chapter 4

Targeting plasma cells with proteasome inhibitors: possible roles in treating myasthenia gravis?

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Abstract:

Myasthenia gravis (MG) is treated primarily with broad-spectrum immunosuppressants, such as prednisone or azathioprine, which normally require several months to reduce auto-antibody titers significantly. This delay may be caused by the resistance of the main antibody-producing cells, the plasma cells, to these drugs. In particular, long-lived plasma cells are resistant to the immunosuppressive treatments and can produce (auto-) antibodies for months. Bortezomib is a proteasome inhibitor approved for treating patients with multiple myeloma, a plasma cell malignancy. Recent preclinical studies in cell cultures and animal models, and clinical studies in organ-transplant recipients, have demonstrated that bortezomib can kill non-neoplastic plasma cells within hours. This suggests that proteasome inhibitors could also be used for rapidly reducing auto-antibody production in autoimmune diseases. We have begun to assess their potential in MG.

Introduction

Myasthenia gravis (MG) is an autoimmune disease characterized by muscle weakness caused by autoantibodies against postsynaptic proteins of the neuromuscular junction. In most MG-patients, they recognize the acetylcholine receptor (AChR) [1], but, in others, the muscle-specific kinase (MuSK) [2] or the low-density lipoprotein receptor-related protein (LRP4) [3] instead. Even though these antibodies cause similar clinical features (muscle weakness and fatigability), they are in fact the result of distinct autoimmune processes. For instance, in early-onset AChR-MG patients, the frequent thymic abnormalities include lymph node-type infiltrates with AChR-specific plasma cells [4, 5], whereas the MuSK-MG thymus hardly differs from that in age-matched controls [6]. Moreover, MuSK-MG is mediated by auto-antibodies of the IgG4 subclass [7], while those in AChR-MG are mainly complement-binding IgG1s and IgG3s [8].

Many aspects of the underlying etiologies of these conditions remain to be defined, but different treatment responses to immunosuppressive drugs and monoclonal antibodies suggest that distinct antibody-producing cell populations are involved [9]. Plasma cells are the main antibody-producing cell type; they can be either short- or long-lived, and they do not divide. Importantly, they survive most immunosuppressive treatments, rendering them relevant targets for therapies aimed at reducing antibody production.

In this review, we focus on the importance of plasma cells as targets for therapy in autoimmune diseases, and on the potential applications of proteasome inhibitors for eliminating these high-rate antibody-producing cells, emphasizing the potential benefits of such therapies for treating MG patients.

Plasma cells in autoimmunity

Plasma cells are one vital end-product of B cell differentiation; unlike their immediate precursor plasmablasts, they do not proliferate or migrate into the circulation. Therefore, they are very rare in the blood and reside predominantly in the bone marrow and secondary lymphoid tissues such as spleen and lymph nodes, where they are embedded in

special niches. Their function is high-rate secretion of antibodies (3700 times more than by B lymphocytes [10]; i.e., >3,000 molecules/cell/second). Consequently, they are essential for maintaining antibody levels in serum and thus for humoral immune responses. Although antibody-producing cells were shown to survive for many months already in the 1960s [11], long-lived plasma cells, and their contribution to humoral memory, have only been described accurately in the last decade [12-14]. Recent studies have demonstrated that their maintenance is largely independent of continuous regeneration from memory B cells [15, 16]. Moreover, the cellular and molecular components of the survival niches, where plasma cells must reside in the longer term, have been defined [17]; they include interactions via VCAM-1/VLA4, CXCL12/CXCR4 and IL-6/IL-6R [18]. While differentiating terminally, plasma cells almost completely downregulate most B cell markers (CD19, CD20, CD22 and MHC class II), though most do express CD38 and CD138. Crucial to their specialization is the upregulation of proteins related to endoplasmic reticulum (ER) stress and the unfolded protein response, including Blimp-1 and XBP1 [19, 20]. The resulting intracellular differentiation is characterized by formation of extensive amounts of rough ER with densely arranged cisternae (Fig. 4.1) typical of professional secretory cells.

In recent years, long-lived plasma cells have proved to play a key role in antibody-mediated autoimmune diseases. For instance, in a mouse model of systemic lupus erythematosus (SLE), they produced substantial amounts of auto-antibodies and were resistant to standard immunosuppression [21]. Furthermore, they appear to maintain the autoimmune response in Sjögren's syndrome [22] and in rheumatoid arthritis patients [23].

Persisting long-lived plasma cells are probably also responsible for the typically slow time course of serum autoantibody decline seen in most MG patients during treatment with steroids and/or other immunosuppressive drugs. Convincing evidence implicating them in MG comes from retrospective studies of rituximab-treated MG-patients. In their long-term follow-up of a cohort of refractory MG-patients treated with the anti-CD20 antibody rituximab, Diaz-Manera et al. showed almost complete elimination of anti-MuSK autoantibodies, whereas anti-AChR levels did not change significantly [9]. Apparently therefore, autoantibodies against MuSK are made mainly by short-lived

plasma cells that are continuously regenerated from autoreactive CD20 positive B cells. By contrast, those against the AChR most likely derive from long-lived plasma cells; so do total circulating IgGs and anti-tetanus toxoid antibodies, which also remained constant after CD20 depletion therapy, again implicating the long-lived plasma cell compartment that is expected to resist it [9]. Similarly, in a randomized phase II/III trial with rituximab in SLE patients [24], autoantibody titers remained unchanged for at least one year, probably because they are maintained by long-lived plasma cells, even if circulating B cells are depleted completely.

Clearly, therefore, drugs targeting plasma cells may have a place in the therapy of autoimmune diseases [25].

Bortezomib targets non-neoplastic plasma cells: *in vivo* studies

Most of the available immunosuppressive drugs are designed to halt proliferation of immune cells, but plasma cells are terminally differentiated and there are very few drugs that target them. Long-lived plasma cells are even resistant to whole-body irradiation [26]. Until recently, their neoplasms (in particular multiple myeloma) were notoriously hard to treat. Even benign plasma cells are typically resistant to most standard immunosuppressant treatments, including such novel drugs as mycophenolate mofetil (MMF) [15, 27, 28], and they express very few targets for therapeutic monoclonal antibodies. At present, one of the most effective FDA-approved drugs for plasma cell malignancies is the proteasome inhibitor bortezomib (also known as Velcade or PS-341). It is currently used for treating patients with mantle cell lymphoma as well as multiple myeloma [29, 30]. However, peripheral neuropathy (PN) is a major side effect of bortezomib – when used repeatedly. PN can be very disabling in up to 30% of treated patients and has seriously discouraged off-label testing of bortezomib in clinical conditions other than cancers. Recent advances in its use (e.g., subcutaneous instead of intravenous administration [31] and lower but effective dose regimens [32]) are showing promise for reducing these risks and thus for broadening its future applicability in other diseases. Recently, a second proteasome inhibitor, carfilzomib (also known as Kyprolis or PR-171), has been approved for the treatment of multiple myeloma. By analogy, it is likely to be

effective against autoimmune plasma cells as well. Since carfilzomib does not increase the risk of PN in multiple myeloma patients [33], this particular side effect of bortezomib may not be proteasome specific [34].

Proteasome inhibition is toxic for myeloma cells because it (a) causes accumulation of misfolded proteins in the ER, triggering the terminal unfolded protein response that ultimately leads to apoptosis [35]; (b) prevents the activation of the transcription factor NF-κB that is crucial for expression of cytokines, chemokines and their receptors, which ensure plasma cell survival [36]. In fact, it is now clear that it is mainly the high-rate production of proteins that sensitizes certain cell types towards proteasome inhibition [37]. That is why Neubert *et al.* predicted that bortezomib would be toxic to non-neoplastic plasma cells too, since they also secrete antibodies in large amounts [38].

Initial studies in animal models demonstrated that proteasome inhibition could indeed kill non-neoplastic plasma cells (Table 4.1). In an SLE model, plasma cells (whether short- or long-lived) were depleted as early as 48 hours after treatment by inducing apoptosis in them [38]. Moreover, long-term treatment (>30 weeks) decreased auto-antibody titers almost to background levels, while approximately halving total IgG levels (possibly reflecting partial recovery of plasma cells after the treatment was stopped). Hence, proteasome inhibition ameliorated the lupus-like nephritis signs in these mice and prolonged their survival. Similarly, we have demonstrated that bortezomib significantly depletes bone marrow plasma cells in an animal model of MG (experimental autoimmune myasthenia gravis, EAMG) [39]. In that study, we treated EAMG-rats with bortezomib twice weekly for four weeks, starting four weeks after immunization with *Torpedo* AChR (by when serum autoantibodies against rat AChR were present). At the end of the experiment, plasma cell numbers and autoantibody titers were significantly lower in bortezomib-treated rats than in saline-injected controls; titers were reduced by 60% after four weeks of treatment. Interestingly, in bone marrow plasma cells, bortezomib induced ultrastructural changes (swelling of the endoplasmic reticulum) characteristic of ER stress (Fig. 4.1*B*, *C*).

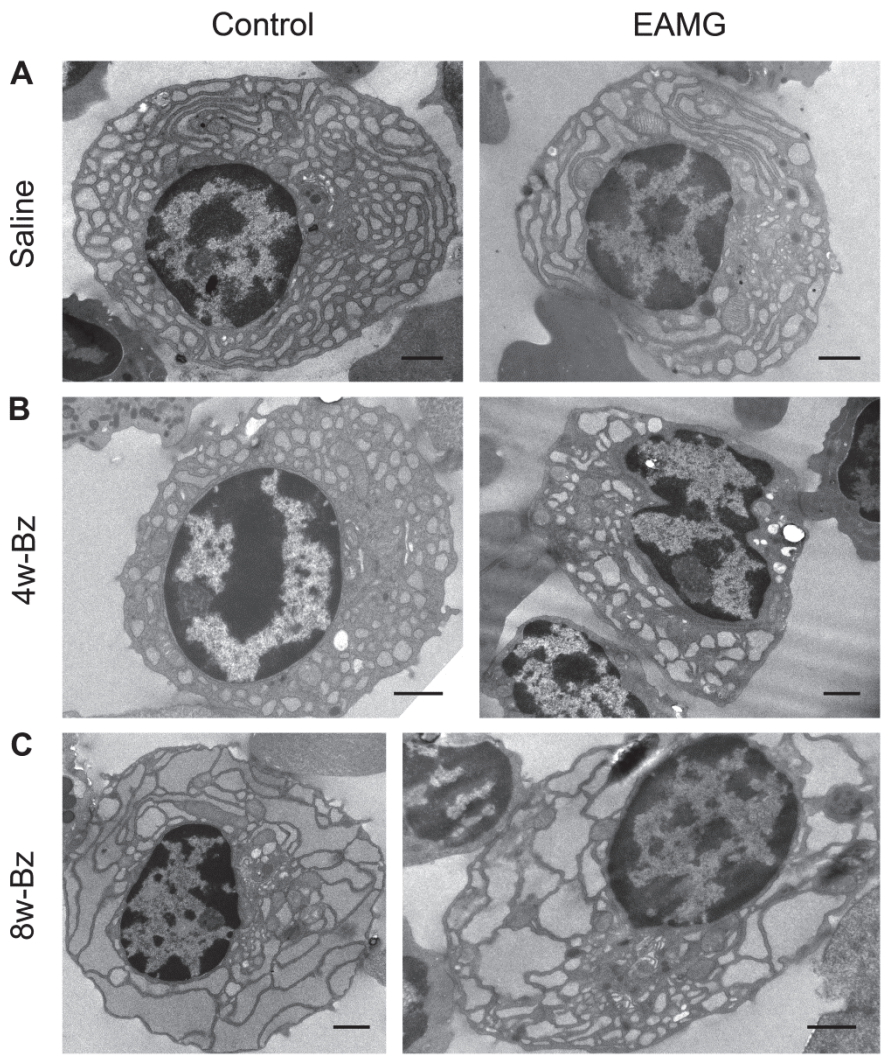

Figure 4.1. Electron micrographs of plasma cells from rat bone marrow. (*A*) Morphologically normal plasma cells after eight weeks of saline treatment. These cells are characterized by an elaborate endoplasmic reticulum (ER), which occupies most of the cytoplasm, and by an eccentric nucleus that contains patches of condensed chromatin (resembling a "cart-wheel" configuration). The Golgi apparatus is easy to distinguish. (*B*) Plasma cells from four-week bortezomib-treated animals. During bortezomib treatment, plasma cells frequently show morphological alterations, including distension of the ER cisternae, with disorganization of its normal structure and derangement of the Golgi complex. (*C*) Plasma cells from eight-week bortezomib-treated animals. Morphological alterations were similar to those in four-week treated animals, with more pronounced dilatation of the ER cisternae. Cells were stained with osmium tetroxide and contrasted with uranyl acetate and lead citrate. Scale bars, 1 µm. Reproduced, with permission, from Reference 39.

Table 4.1. Summary of *in vitro* and *in vivo* studies with bortezomib (Btz) in non-neoplastic models of disease

Reference	Disease model	Btz dose and schedule(s)	Effects on PC	Main findings
***In vitro* studies**				
Perry et al. [27]	PC from the BM of renal-transplant recipients	1 mg/L for 24 h or 72 h	Btz induced apoptosis in more than 60% of PC already after 24 h	Btz, but not Rtx or IVIg, blocked the production of allo-antibodies by PC after 72 hours. A dose-response curve showed that Btz is effective at concentrations ≥1 mg/L
Gomez et al., unpublished data	Thymic cells from MG patients	1 mg/L twice (days 7 and 11) in a 14-day culture schedule	No Btz-exposed PC survived to day 14. In cultures with Dx, Len or saline, significantly more PC survived until day 14	Autoantibody and IgG production were completely blocked by Btz, but not by Dx, Len, or saline. On dose-response testing, Btz eliminated PC at concentrations ≥4 μg/L
***In vivo* studies**				
Neubert et al. [38]	SLE-like mice (NZB/W F1; MRL/lpr)	0.75 mg/kg - Twice at 36 h interval (A) - 3 times in 1 week (B) - Twice weekly for 40 weeks (C)	>60% reduction of both short- and long-lived PC in the BM, from 48 h after the first dose (A)	Significant reduction of anti-ds-DNA antibody titers only with Btz treatment (not Dx or Cy) (B). Long-term treatment with Btz improved the clinical condition of these mice (C)
Gomez et al. [39]	EAMG rats	0.2 mg/kg - For prevention: twice weekly for eight weeks (8w-Btz) - For treatment: four weeks Btz, starting four weeks after induction of the model (4w-Btz)	Strong reduction of total PC in the BM, with both regimens	Significant reduction of anti-AChR antibody titers and total IgG levels in both the 8w-Btz and 4w-Btz groups, i. e., also when autoantibodies were already present (4w-Btz)

Continued

Table 4.1. *Continued*

Reference	Disease model	Btz dose and schedule(s)	Effects on PC	Main findings
Vogelbacher et al. [40]	Chronic allograft nephropathy model in rats	0.2 mg/kg - Twice weekly for nine weeks	Significant depletion of PC in the BM and the spleen	Significant reduction of allo-antibody titers and IgG-producing cells. Some additive actions were suggested for Btz and the mTOR-inhibitor sirolimus
Meslier et al. [41]	Experimental hemophilia-A mice that develop anti-factor VIII antibodies	0.75 mg/kg - For prevention: Btz twice weekly for four weeks - For treatment: Btz twice weekly for 10 weeks	Both short- and long-lived PC were strongly reduced by Btz in the spleen, but not in the BM, with the preventive schedule. The number of autoreactive PC in BM was not reduced by Btz treatment	Btz was effective in preventing anti-factor VIII antibody responses but hardly reduced them once they were established
Ichikawa et al. [42]	SLE-like mice (NZB/W F1, ONX 0914, NZB/NZW)	0.5 mg/kg - Twice weekly for 13 weeks	Btz decreased by >90% the number of PC in the spleen and the BM	Greater sensitivity of autoantibody- than total IgG- secreting PC to proteasome inhibition. New proteasome inhibitors (carfilzomib and ONX 0914) also gave positive results
Bontscho et al. [43]	Mouse model of vasculitis due to anti-neutrophil cytoplasmic antibodies (ANCA)	0.75 mg/kg twice weekly - Early treatment: 5 weeks of Btz, starting 3 weeks after induction of the model - Late treatment: 3 weeks of Btz, starting 5 weeks after induction of the model	Spleen and BM PC were significantly reduced by Btz. Auto-reactive PCs were more susceptible to Btz treatment	Btz significantly reduced autoantibody titers already one week into early treatment, and protected against development of clinical signs. Late treatment reduced antibody titers at the endpoint but did not improve the clinical condition. Combined steroid/Cy significantly reduced PC numbers in the spleen, but not in BM

AMR, antibody-mediated transplant rejection; BM, bone marrow; Btz, bortezomib; Cy, cyclophosphamide; Dx, dexamethasone; EAMG, experimental autoimmune myasthenia gravis; IVIg, intravenous immunoglobulin; Len, lenalidomide; PC, plasma cell; Rtx, rituximab.

Bortezomib is toxic for non-neoplastic plasma cells: *in vitro* studies

Recent *in vitro* studies have focused on the sensitivity of nonmalignant plasma cells to proteasome inhibition (Table 4.1) [27, 38-43], and especially – in antibody-mediated kidney transplant rejection – on its potential for depleting allo-reactive (anti-HLA) plasma cells. In one of the first such studies [27], bortezomib, but not rituximab or IVIg, induced apoptosis within 72 hours in plasma cells cultured from the bone marrow of transplant recipients. In addition, it completely blocked allo-antibody production in these cultures, while rituximab had no significant effect. These results, along with pilot clinical studies *in vivo* [44], prompted the off-label use of bortezomib for preventing transplant rejection in patients, as discussed later in this review.

To extend this approach to autoimmune diseases, we took advantage of the very prevalent lymph node-like infiltration into the early-onset MG thymus [4-6, 45, 46], where germinal centers form close to rare muscle-like myoid cells [4], and plasma cells spontaneously secrete anti-AChR antibodies [5, 45, 46]. Since the thymus is removed surgically in many of these patients as part of treatment, it is an accessible and valuable source of specific autoreactive plasma cells for research [5]. In culture, these continue spontaneously secreting substantial amounts of AChR autoantibodies for several weeks (at least) – even after irradiation, a feature unique to plasma cells [5]. They are apparently sustained by “feeder effects” from accessory thymic cell types (e.g., fibroblasts and macrophages) [5, 45, 46].

We therefore exploited this source of autoimmune plasma cells as a test-bed for evaluating their sensitivity to drugs used against myelomas. These included bortezomib, dexamethasone, and the thalidomide derivative lenalidomide (now also approved for myeloma therapy); we measured (auto-) antibody and total IgG production and plasma cell survival after two weeks in culture (unpublished results). Bortezomib proved to target plasma cells with surprising efficiency; they were already almost undetectable after 24 h, and even at 60 times lower concentrations than those reached in patients’ plasma [31]. Plasma cells appeared apoptotic as soon as eight hours after adding bortezomib to the cultures. Moreover, autoantibody and total IgG production were completely blocked upon addition of bortezomib, whereas dexamethasone caused a mild but statistically significant

reduction and lenalidomide had no significant effect. Importantly, at the doses used, none of these drugs caused extra death of thymic cells by day 14 (i.e., above the rate of apoptosis expected of thymocytes). Thus, bortezomib's toxicity seems selective for plasma cells.

Experience with bortezomib in transplantation and autoimmune disorders

Since its introduction, there have been many clinical trials and case reports of bortezomib's benefits and side effects in multiple myeloma patients [47], but few on its off-label use in non-neoplastic diseases (Table 4.2) [44, 48-52]. However, there are reports of positive outcomes in the treatment of autoimmune hemolytic anemia [48, 53], rheumatoid arthritis [54], and SLE [55]. The most extensive studies have focused on the prevention of antibody-mediated rejection in renal transplant recipients [56]. Initially, bortezomib was only used as a last resort in patients who were refractory to several immunosuppressive drugs [44]; subsequently, in combination with plasma exchange, it also proved effective as primary treatment for rapidly reducing anti-HLA antibody levels in hyper acute transplant rejection [57]. Bortezomib reduced both numbers of allo-reactive plasma cells in the bone marrow and serum allo-antibody titers in strongly allo-sensitized renal patients prior to transplantation [49]. Moreover, it significantly increased graft survival in comparison to rituximab [52]. However, despite these positive findings, there is one other report that a single cycle of bortezomib as a monotherapy was not beneficial in sub-acute rejection when given after transplantation [51]. Moreover, it seems more effective in early than in late acute rejection [58].

By contrast, there is much less published experience with proteasome inhibition in autoimmune diseases. To date, two reports have described a reduction of autoantibody titers and a clear increase of hemoglobin levels in cases of steroid/rituximab-refractory autoimmune hemolytic anemia [48, 53]. In addition, in one multiple myeloma patient, the coincidental SLE improved substantially and anti-dsDNA titers declined significantly after just 3 cycles of bortezomib [55]. Similarly, Hiepe *et al.* recently reported significant clinical improvements and autoantibody titer reductions in four patients with refractory SLE after

treatment with bortezomib plus dexamethasone [59]. Protective vaccine-specific antibodies and total IgG levels were also decreased in these patients, suggesting that long-lived plasma cells were indeed depleted by proteasome inhibition. However, since repeated treatments were necessary to achieve sustained responses, combinations of bortezomib with agents that can also eliminate plasma cell precursors (i.e., rituximab) were recommended.

Table 4.2. Summary of clinical studies with bortezomib in transplantation and autoimmune disorders

Reference	Patients	Btz dose and schedule(s)	Main findings
Carson et al. [48]	Case report of refractory IgM-mediated autoimmune hemolytic anemia	1.3 mg/m^2 - Three doses of Btz	Improvement of symptoms and increase of hemoglobin levels after Btz treatment. IgM autoantibodies were significantly decreased
Hiepe et al. [59]	Four refractory SLE patients	1.3 mg/m^2 - Four cycles of Btz (days 1, 4, 8, 11) with 10-day treatment-free intervals - 20 mg Dx was given in combination	Significant clinical improvement and reduction of lupus-associated antibodies. Total IgG levels and vaccine-specific antibodies were decreased, suggesting that the bone marrow PCs were affected. The authors recommend a combination therapy of Btz with B cell depletion to ensure long-lasting improvement
Everly et al. [44]	Six renal transplant recipients with refractory AMR	1.3 mg/m^2 - One cycle of Btz (days 1, 4, 8, 11) - PE and Rtx were sometimes used as adjuvant therapy	Reversion of transplant rejection, amelioration of the clinical condition, and reduction of allo-antibody titers. Effects were sustained for at least five months. No severe side effects reported
Walsh et al. [58]	30 renal transplant recipients with AMR. 13 had early onset AMR (< six months post-transplant) and 17 had late onset AMR (> six months post-transplant)	1.3 mg/m^2 - One cycle of Btz (days 1, 4, 8, 11) - PE and a single dose of Rtx were used as adjuvant therapies	Btz improved renal function and reduced allo-antibody titers in both early and late AMR, but more so in early than late AMR. Peripheral neuropathy was observed more frequently in late-onset AMR. There were no reports of severe cases (grades 3 or 4)
Sberro-Soussan et al. [51]	Four renal transplant recipients with AMR	1.3 mg/m^2 - One cycle of Btz (days 1, 4, 8, 11) plus maintenance immunosuppressive treatment	After five months' follow-up, no reductions in allo-antibody or IgG levels were observed. Bilateral conjunctivitis and prolonged fatigue were reported as severe side effects

Continued

Table 4.2. *Continued*

Reference	Patients	Btz dose and schedule(s)	Main findings
Diwan et al. [49]	Eight sensitized renal-transplant candidates, with high anti-HLA antibody titers	1.3 mg/m^2 - One or four cycles of Btz (4 or 16 doses)	Monotherapy with Btz significantly reduced the number of both tetanus-specific and anti-HLA PC in the bone marrow of patients. Total PC numbers were also reduced by Btz, but not significantly. Treatment with Btz prolonged the reduction of anti-HLA antibodies by PE. No randomized paired-control group of bone marrow aspirates was included on this study
Everly et al. [50]	13 renal transplant recipients with refractory AMR	1.3 mg/m^2 - One or two cycles of Btz - PE and methylprednisone were used as adjuvant therapies	Anti-HLA antibody levels were reduced by ≥50% after one month of treatment; after one year, antibody titers were still low. Total IgG, antibodies against measles and tetanus toxoid remained unchanged, and at protective levels, at one year post-treatment. The authors suggest that the lower antibody production of protective PC makes them less susceptible to proteasome inhibition
Waiser et al. [52]	10 renal transplant recipients with AMR	1.3 mg/m^2 - One cycle of Btz (days 1, 4, 8, 11) - PE and IVIg were used as adjuvant therapies	Outcomes were compared to a historical group of patients treated with the same adjuvant therapy but with one single dose of Rtx instead of Btz. At 18 months post-treatment, graft survival was superior in Btz-treated patients. Both Btz and Rtx significantly reduced peak anti-HLA antibody levels during the follow-up period, but neither eradicated existing allo-antibodies

AMR, antibody-mediated transplant rejection; Btz, bortezomib; Dx, dexamethasone; IVIg, intravenous immunoglobulin; PC, plasma cell; PE, plasma exchange; Rtx, rituximab; SLE, systemic lupus erythematosus.

Proteasome inhibitors for myasthenia gravis patients?

Generally speaking, chronic autoimmune diseases such as MG can seriously affect quality of life because of persistence of clinical symptoms and/or side effects of long-term

medication (e.g., diabetes, hypertension and osteoporosis caused by glucocorticoids [60]). Broad-spectrum immunosuppressive drugs, such as prednisone and azathioprine, ameliorate the symptoms in most MG patients. However, there is typically a long delay (six to eight weeks for steroids [60] and up to 18 months for azathioprine [61]) before autoantibody titers decline and muscles strengthen significantly. That might well be because of treatment-resistant plasma cells. If so, their specific elimination could represent a novel and promising approach for treating MG. In time, we hope that there will be biomarkers to predict which MG patients will respond rapidly to standard immunosuppressants and which will need adjunctive plasma cell depletion to achieve earlier improvements, whether in acute severe or chronic refractory cases.

An important limitation in using bortezomib in nonmalignant diseases is the concern about development of PN, which is a serious but generally reversible side effect [62] that seems to depend on reaching a cumulative bortezomib-dose threshold during the first five cycles of treatment [63]. Whereas current regimens were designed for multiple myelomas (which are notoriously hard to treat), lower doses and short courses might be sufficient to deplete autoreactive plasma cells significantly, e.g., in MG. For instance, Diwan *et al.* showed that four cycles of bortezomib were already sufficient to significantly deplete bone marrow plasma cells in transplant recipients [49]. In a large clinical trial in myeloma patients, single weekly doses proved as effective as the established twice weekly schedule, with significantly lower risks of both any-grade PN (35% vs. 51%) and grade 3/4 PN (8% vs. 28%) [32]. In addition, using the recently FDA-approved subcutaneous (instead of intravenous) route reduced the incidence and severity of PN (any grade PN 38% vs. 53%; grade 3/4 PN 6% vs. 16%) [31]. Furthermore, second-generation proteasome inhibitors are now approved (carfilzomib) or being developed (some of them are already in use in clinical trials); they seem to be more selective than bortezomib and potentially less neurotoxic [34, 64]. Another interesting possibility is to combine bortezomib with other drugs now being used for treating myelomas. These include the immunomodulatory drugs thalidomide and lenalidomide, drugs blocking the interactions that maintain plasma cells in their survival niches in the bone marrow (natalizumab [65] against VLA-4, tocilizumab [66] or siltuximab [67] against IL-6, and

plerixafor [68] against CXCR4), or drugs reactive with plasma cells directly (elotuzumab [69] against CS1, daratumumab [70] against CD38, or nBT062 [71] against CD138).

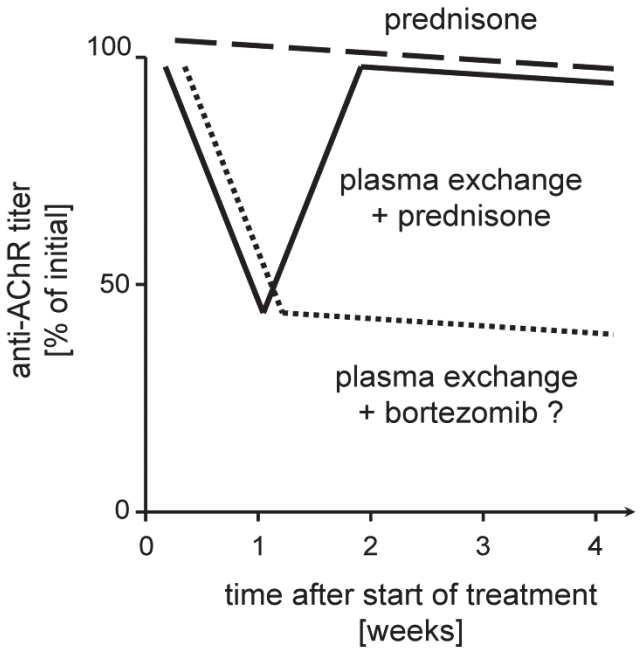

Figure 4.2. Schematic representation of predicted changes in anti-AChR antibody titers following different immunosuppressive treatments. Initial treatment with prednisone only (dashed line) normally requires several weeks to reduce autoantibody titers significantly. Plasma exchange (solid line) reduces them rapidly, but – even in patients also taking prednisone and other immunosuppressive drugs – titers return almost to their initial levels within the next few weeks [72]. The combination of plasma exchange with bortezomib (dotted line) could induce a more lasting reduction in antibody titers, based on results of such a treatment in organ transplant recipients [50].

Conclusions

In conclusion, proteasome inhibition has significant potential as an effective induction therapy, in combination with plasma exchange, for MG patients with high autoantibody titers and/or acute severe weakness. Removing autoreactive plasma cells should lead to more sustained reductions in autoantibody titers after plasma exchange (Fig. 4.2), a procedure which, by itself, leads to only temporary reductions in autoantibodies in the plasma [72]. Additional immunosuppression with glucocorticoids (at lower than normal doses), azathioprine, MMF, or rituximab would probably be necessary to prevent

generation of new autoreactive plasma cells – by when bortezomib will have served its purpose. Moreover, it might prove valuable in refractory MG.

To summarize, we consider that proteasome inhibition represents a promising new approach for MG treatment. Experience from animal models, from *in vitro* settings and from transplant recipients convincingly supports the potential of bortezomib for targeting plasma cells, the main antibody-producing cells. Naturally, future work is needed to confirm the potential of proteasome inhibition in antibody-mediated autoimmune diseases.

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Chapter 5

Proteasome inhibition with bortezomib depletes plasma cells and autoantibodies in experimental autoimmune myasthenia gravis

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Abstract

Bortezomib, an inhibitor of proteasomes, has been reported to reduce autoantibody titers and to improve clinical condition in mice suffering from lupus-like disease. Bortezomib depletes both short- and long-lived plasma cells; the latter normally survive the standard immunosuppressant treatments targeting T and B cells. These findings encouraged us to test whether bortezomib is effective for alleviating the symptoms in the experimental autoimmune myasthenia gravis (EAMG) model for myasthenia gravis, a disease that is characterized by autoantibodies against the acetylcholine receptor (AChR) of skeletal muscle. Lewis rats were immunized with saline (control, *n* = 36) or *Torpedo* AChR (EAMG, *n* = 54) in complete Freund's adjuvant (CFA) in the first week of an experimental period of eight weeks. After immunization, rats received twice a week subcutaneous injections of bortezomib (0.2 mg/kg in saline) or saline injections. Bortezomib induced apoptosis in bone marrow cells and reduced the amount of plasma cells in the bone marrow by up to 81%. In the EAMG animals, bortezomib efficiently reduced the rise of anti-AChR autoantibody titers, prevented ultrastructural damage of the postsynaptic membrane, improved neuromuscular transmission, and decreased myasthenic symptoms. This study thus underscores the potential of the therapeutic use of proteasome inhibitors to target plasma cells in antibody-mediated autoimmune diseases.

Introduction

The resistance of long-lived plasma cells against immunosuppressive medication poses a serious problem for the treatment of antibody-mediated autoimmune diseases. Currently used immunosuppressive drugs, including corticosteroids, mitomycin C, cyclosporine A, azathioprine, and cyclophosphamide, affect mainly activated and dividing B and/or T cells [1]. Plasma cells are the terminally differentiated, non-dividing effector cells of the B cell lineage that have lost many surface markers. In their survival niches in the spleen and in particular in the bone marrow [2], resident long-lived plasma cells are resistant to most therapies including immunosuppressive drugs and anti-CD20 antibodies that are aimed to inhibit the activation and/or proliferation of lymphocytes or to deplete certain lymphocyte subpopulations [3, 4]. Thus, resistance to available therapies might be due to persistent long-lived plasma cells that continue to produce autoantibodies notwithstanding immunosuppressive treatment [5].

However, because of their high rate of immunoglobulin production, both short- and long-lived plasma cells are particularly sensitive to inhibition of the proteasome [6, 7]. Indeed, proteasome inhibition causes accumulation of non-degraded, misfolded proteins within the endoplasmic reticulum (ER) of plasma cells and, subsequently, to activation of the terminal unfolded protein response, ultimately leading to apoptosis [8]. The proteasome inhibitor bortezomib, also known under the trade name Velcade, is a boronic acid dipeptide (phenylalanine-leucine) derivative, which binds reversibly to the 26S proteasome [9]. After injection, bortezomib is distributed widely and quickly to the blood and most tissues [10]. Currently, bortezomib is approved for the treatment of multiple myeloma and mantle cell lymphoma. In addition to the treatment of B cell malignancies, proteasome inhibition could be a useful therapeutic strategy for antibody mediated autoimmunity such as lupus [7].

In the present study we tested the effect of proteasome inhibition in a model for myasthenia gravis (MG), a well-characterized disease that is found to be critically dependent on the level of autoantibodies against the acetylcholine receptors (AChRs) of muscle. In 85% of MG patients the disease is caused by autoantibodies against the muscle

AChR [11]. The remaining patients have autoantibodies against the muscle specific kinase (MuSK) [12] (~ 5% of all MG patients) or no detectable autoantibodies (idiopathic MG, accounting for ~ 10% of all patients) [13]. Both AChR and MuSK proteins are located in the postsynaptic membrane of the neuromuscular junction (NMJ), which is specialized to respond to the neurotransmitter acetylcholine released from the overlying nerve ending. These proteins are thus essential for muscle contraction, and MG is potentially fatal, since autoantibodies can cause respiratory failure by impairing neuromuscular transmission. In such an event of acute myasthenic crisis, the first choice of treatment is plasma exchange, mechanical ventilation [14], and intravenous treatment with high doses of IgG [15]. Plasma exchange typically leads to significant improvement or remission within a few days in most patients, including those with the idiopathic MG, but the effect is, of course, not long-lasting. Immunosuppressive drugs such as prednisone and azathioprine are generally used for long-term therapy [1, 16-18]. Because these depend mostly on preventing the activation, proliferation, and differentiation of developing B and T cells, the autoantibody titers only drop over a period of months. Using an established immunotherapy protocol with prednisone and azathioprine, it may take as much as 18 months before patients improve [16]. For the intermediate time interval, during which plasma cells continue to produce autoantibodies, proteasome inhibition might be a useful therapy. Moreover, because some MG patients do not respond well to any currently available treatment in terms of poor reduction of autoantibodies or the occurrence of side effects, proteasome inhibition might provide a therapeutic alternative.

In the experimental autoimmune myasthenia gravis (EAMG) model the disease is induced by immunizing rats with the AChR from the electric organ of the electric ray *Torpedo californica* [19, 20]. A small proportion of antibodies against the *Torpedo* AChR cross-reacts with the AChR of the muscle [21]. As in human MG with anti-AChR autoantibodies, the disease symptoms in EAMG are caused by antibody-mediated destruction of the NMJ [22, 23].

In this study, we examined the effect of bortezomib in EAMG rats using two different treatment regimes. The first treatment consisted of bortezomib injections for eight weeks, starting directly after immunization (herein referred to as 8w-Bz). For the

second treatment regime, rats were injected with bortezomib starting only four weeks after immunization, when autoantibody titers were already detectable, until eight weeks after immunization (4w-Bz). Using this setup we investigated the potential therapeutic effect of bortezomib after onset of the disease. The results show that both treatment regimes reduce autoantibody levels by depleting bone marrow plasma cells, but only the 8-week bortezomib-treatment led to a significant improvement of the clinical condition of the EAMG rats and to a reduction of postsynaptic damage.

Materials and Methods

Animals

Inbred female Lewis rats (*n* = 90) were obtained from the Department of Experimental Animal Services, Maastricht University (The Netherlands). Permission to perform this experiment was granted by the Committee on Animal Welfare, according to Dutch governmental rules. At 6 weeks of age, animals were weighed and divided into six experimental groups (Table 5.1) with an equal average weight.

Induction of EAMG

EAMG was induced in 7-week-old rats (*n* = 54) by active immunization with AChR purified from the electric organ of *T. californica* (tAChR) in complete Freund`s adjuvant (CFA). In brief, 20 µg of tAChR [20] was dissolved in 100 µL of phosphate-buffered saline (PBS) and emulsified with an equal volume of CFA with 0.1% of *Mycobacterium tuberculosis* H37 (Difco Laboratories, Detroit, MI). Animals were initially anesthetized in a cylindrical tube through which 5% isoflurane in air was supplied. Subsequently, 3% isoflurane was supplied by a cap over the head and 200 µL CFA/tAChR emulsion was injected subcutaneously at the base of the tail at three different spots, as described by Lennon and colleagues [24]. Control animals (*n* =36) were injected with an equal volume of emulsified PBS and CFA. Rats were sacrificed eight weeks after immunization or earlier, if they had reached the humane endpoints as described below. They were sacrificed by inhalation of CO_2 in air and subsequent cervical dislocation.

Experimental design and administration of drugs

Bortezomib was purchased as a lyophilized powder (Velcade, Janssen-Cilag B.V., Beerse, Belgium) and dissolved in sterile saline solution at a final concentration of 0.1 mg/mL. Two weekly doses of 0.2 mg/kg bortezomib solution were administered subcutaneously, which is considered the highest dose to use without having increased mortality rates or severe side effects in rats [10, 25, 26]. Control and EAMG groups were subdivided into three treatment regimes each (Table 5.1). "Saline" groups received two weekly subcutaneous injections of 0.9% NaCl solution (2 mL/kg) at the neck for eight weeks. The 4w-Bz groups received two weekly saline injections for the first four weeks after immunization (by which time autoantibody levels were highly elevated in the plasma) and subsequently two weekly injections of bortezomib for another four weeks. The 8w-Bz groups received two weekly injections of bortezomib for eight weeks, starting directly after immunization.

For practical reasons the experiment was conducted three times in sets of 30 animals each, including an equal number of all the aforementioned groups. The animals' tissues were analyzed using electron microscopy (EM), electromyography (EMG), immunofluorescence (IF), and fluorescence activated cell sorting (FACS), and the number of animals used for each method is indicated in Table 5.1.

Table 5.1. Treatment groups

	Saline groups (no bortezomib)	**4w-Bz groups (bortezomib started 4 weeks after immunization)**	**8w-Bz groups (bortezomib started directly after immunization)**
Control (immunized with CFA)	*n* = 12 (IF and FACS, *n* = 5; EM and EMG, *n* = 7)	*n* = 12 (IF and FACS, *n* = 5; EM and EMG, *n* = 7)	*n* = 12 (IF and FACS, *n* = 5; EM and EMG, *n* = 7)
EAMG (immunized with AChR in CFA)	*n* = 18 (IF and FACS, *n* = 8; EM and EMG, *n* = 10)	*n* = 18 (IF and FACS, *n* = 8; EM and EMG, *n* = 10)	*n* = 18 (IF and FACS, *n* = 8; EM and EMG, *n* = 10)

CFA: Complete Freund's adjuvant; IF: immunofluorescence; FACS: fluorescence activated cell sorting; EM: electron microscopy

Weight and clinical scoring

The weights of animals were measured on a weekly basis as a general indicator of health and for dose calculations. The severity of clinical signs of EAMG was scored weekly by measuring muscular weakness by two blinded investigators (A.M.G. and M.P.). The animals' muscle strength and fatigability was assessed by their ability to grasp and lift repeatedly a 300-g metal rack from the table, while suspended manually by the base of the tail for 30 seconds [27-29]. Clinical scoring was based on the presence of tremor, hunched posture, muscle strength, and fatigability. Disease severity was expressed as follows: 0, no obvious abnormalities; 1, no abnormalities before testing, but reduced strength at the end; 2, clinical signs present before testing, that is, tremor, head down, hunched posture, weak grip, difficulty in breathing; 3, severe clinical signs present before testing, no grip, moribund [24].

Animals that reached a clinical score of level 3, or lost more than 20% of their weight in the course of one week, were sacrificed within 24 h.

Tissue preparation

For EM analysis, bone marrow was extracted from the femurs by cutting off their upper and lower endings and flushing the shaft with 10 mL sterile saline solution through the bone marrow cavity using a syringe with a 25-gauge needle. For FACS analysis of bone marrow, PBS containing 2% fetal calf serum and 0.1% NaN_3 (FACS buffer) was used instead. Thymus and spleen tissue were entirely removed from the animals by dissection and the organ weight was measured. Single cell suspensions were prepared using a gentleMACS tissue dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Cells of different tissues were resuspended in FACS buffer and run through a 70-µm nylon cell strainer to remove large cell clumps. Numbers of viable cells were determined by counting trypan blue negative cells on hemocytometers. Cells were divided into microcentrifuge tubes (10^5 cells/100 µL/tube) and then stained for dead cells, T cells, and B cells.

Heparinized blood samples were taken from the vena saphena weekly, starting

on the week before immunization (week 0) until the last week of experiment (week 8). Peripheral blood mononuclear cells (PBMCs) were isolated by lysing red blood cells using FACS lysing buffer (BD Biosciences). PBMCs from 80 µL blood were divided into microcentrifuge tubes in a ratio of 20/20/40 µL to stain for dead cells, T cells, and B cells, respectively.

Proteasome activity assay

The proteolytic activity of the proteasome was evaluated in whole blood [30] by means of a 20S proteasome activity kit (APT 280; Millipore), as described by the manufacturer. Taking into account the pharmacokinetic and pharmacodynamic profiles of subcutaneous bortezomib administration [31], blood samples were obtained between 3 and 6 h after administration of bortezomib or saline. In brief, 80 µg whole blood protein extract was incubated in the provided buffer with 3.8 µg fluorophore-linked peptide substrate (LLVY-7-amino-4-methylcoumarin [AMC]) for 120 min at 37ºC. Proteasome activity was measured by quantification of relative fluorescent units from the release of the fluorescent cleaved product AMC using a 380/460 nm filter set in a fluorometer (Victor X3 multilabel reader, PerkinElmer). A solution of the 20S proteasome subunit and the proteasome inhibitor lactacystin were used as controls for the assay.

Autoantibody titers

Antibody titers against rat AChR were measured in plasma samples with an immunoprecipitation radioimmunoassay (RIA). In brief, 2.5 µL of plasma was incubated at 4ºC overnight with 100 µL rat muscle cell-membrane extract (containing ~5 nmol/L AChR; isolated from denervated rat muscles). The AChR was labeled with an excess of [^{125}I]-α-bungarotoxin (NEX126, 5 TBq/mmol; PerkinElmer). The immune-complexes were precipitated by addition of 150 µL goat anti-rat IgG serum and incubation for 4 h at 4ºC. Pellets were washed three times in PBS with 0.5% Triton X-100 and centrifuged at 25,000 X *g* for 5 min. Radioactivity was measured on an automatic gamma counter (2470 Wizard2; PerkinElmer). Autoantibody titers were expressed as nanomoles of α-bungarotoxin binding sites per liter.

Total IgG ELISA

A sandwich ELISA was used to measure total IgG content in plasma samples. Briefly, ELISA plates (Microlon 655092, Greiner Bio-One, Frickenhausen, Germany) were coated with 50 µL catching antibody (goat Ig anti-rat IgG, ab6252, Abcam, Cambridge, U.K.), diluted 1:200 in coating buffer (50 mM sodium carbonate [pH 9.6]), for one h at 37ºC. Plates were washed with PBS containing 0.05% Tween 20 and blocked for 30 min with 100 µL 4% nonfat dry milk in PBS (blotting grade blocker, catalogue no. 1706404; Bio-Rad). Afterwards, 50 µL plasma samples, diluted 1:20,000 in incubation buffer (PBS plus 1% BSA plus 0.02% Tween 20), were incubated for 1 h at 37ºC. A standard curve was made using serially diluted samples of purified rat IgG (catalogue no. I8015; Sigma-Aldrich). After washing, 50 µL HRP-conjugated antibody (ab6257; Abcam), diluted 1:5000 in incubation buffer, was added and plates were incubated for another h at 37ºC. Following a washing step, 100 µL tetramethylbenzidine substrate solution (s(HS)TMB; SDT Reagents, Baesweiler, Germany) was used to develop HRP-labeled antibodies bound to the plates. The color reaction was allowed to develop for 10 min and stopped with 50 µL 2 M sulphuric acid. The optical density was measured at 450 nm filter using a microplate reader (Victor X3 multilabel reader). Results were expressed as milligrams of total IgG per milliliter of plasma.

Apoptosis assays

Early apoptotic and dead cells were identified by flow cytometric analysis according to surface binding of FITC-labeled annexin V to exposed membrane phosphatidylserine and propidium iodide (PI) staining (annexin V-FITC apoptosis detection kit; BD Biosciences, Breda, The Netherlands). The cells (10^5/100 µL) were washed with annexin V-binding buffer and incubated with 5 µL annexin V and 5 µL of PI for 15 min at room temperature. Without washing, cells were immediately measured. Annexin V^+/PI^- cells were regarded as early apoptotic, while annexin V^+/PI^+ cells were considered dead cells.

Extracellular staining for T cell markers

The cells were washed once with FACS buffer by centrifugation at 250 X *g* at 4ºC and

incubated for 30 min at 4ºC with antibodies directed against CD3 (FITC-labeled), CD4 (PE-labeled), and CD8 (PerCP-labeled) (all from BD Biosciences), diluted 1:50 in FACS buffer. The samples were washed twice and kept at 4ºC in the dark until measurement within 2 h.

Intracellular staining for B cell markers

Cells were washed once with FACS buffer by centrifugation at 250 X *g* at 4ºC and incubated with PE-conjugated antibody against CD45RA (BD Biosciences), diluted 1:20 in FACS buffer. After one washing step, cells were fixed with 2% paraformaldehyde in FACS buffer for 10 min at 37ºC. After two more washing steps, cells were permeabilized with cold 90% methanol for 30 min on ice. Cells were washed twice and incubated with FITC-conjugated antibody directed against intracellular immunoglobulin kappa (Igκ) (BD Biosciences), diluted 1:20 in FACS buffer. The samples were washed twice and kept at 4ºC in the dark until measurement within 2 h. Because no appropriate CD markers were available to specifically detect rat plasma cells, we measured rat plasma cells by using high levels of intracellular Igκ expression.

FACS analysis

All cytofluorometric analyses were performed on a FACSCalibur (BD Biosciences) and analyzed using the CellQuest software (BD Biosciences). Forward and sideward light angle scatters were collected. Using these plots, samples were gated to exclude cell debris and cellular aggregates for further analysis. For each marker, the percentage of positive cells stained above background was measured for all gated cells. The cutoff was defined using unstained cells processed alongside the experimental samples.

Plasma cell quantification by EM

Bone marrow cell suspensions were fixed by mixing with an equal volume of 5% glutaraldehyde in PBS and then centrifuged at 800 X *g*. Pellets were resuspended in PBS and embedded in 10% gelatin, then centrifuged again at 1000 X *g* to form a compact pellet. Cell pellets were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) dehydrated through a graded ethanol series and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections from whole pellets were

contrasted with uranyl acetate and lead citrate and viewed with a Philips CM 100 electron microscope. Plasma cell recognition was based on morphological features that are particular to this cell type, such as the typical 'cartwheel' chromatin configuration in the nucleus and the presence of extensive rough ER in the cytoplasm, indicative of an intense protein production. Quantification was performed at six different portions of the sample to have a systematic representation of the cell gradient in the sample that resulted from the centrifugation. Results are expressed as percentage of plasma cells; a total of 800 bone marrow cells were counted per sample.

Electromyography

Animals were initially anesthetized as described above and subsequently intubated in the trachea. Anesthesia was maintained with 3% isoflurane in air. Compound muscle action potential (CMAP) decrement was measured in the tibialis anterior muscle using the EMG system Viking IV (Nicolet Biomedicals, Madison, WI) at the end of the experimental period. For stimulation, two small monopolar needle electrodes were used. The cathode was inserted near the peroneal nerve at the level of the knee and the anode more proximal and lateral at a distance of 3–4 mm. For recording, a third monopolar needle electrode was inserted subcutaneously over the tibialis anterior muscle. The reference electrode was inserted subcutaneously near the ankle. A ring electrode around the tail served as ground electrode. To detect a decremental response of the CMAP, series of eight supramaximal stimuli were given at 3 Hz with 0.2 ms duration. The test was considered positive for decrement when both the amplitude and the area of the CMAP-negative peak showed a decrease of at least 10% [32]. To demonstrate reproducibility, at least three consecutive decrement recordings were made of all investigated muscles. During the measurements, skin temperature was kept between 35°C and 37°C by means of an infrared heating lamp. If initially no decrement was present in the tibialis anterior muscle, neuromuscular transmission was challenged by a continuous intravenous infusion of curare [(+)-tubocurarine, T2379; Sigma-Aldrich]. A solution of 20 µg/mL curare was injected into the vena saphena using a Terfusion syringe pump (model STC-521; Terumo, Tokyo, Japan) at a rate of 1 mL/h (0.33 µg curare/min). During curare infusion, CMAP

measurements were repeated with intervals of 1 min until a repeated decrement was observed. The resistance against curare was used as an indirect, albeit nonlinear, measure for the safety factor of neuromuscular transmission and thus for the performance of neuromuscular transmission [33, 34].

EM of muscle tissue

Electron micrographs were taken from endplates of the tibialis anterior muscles. Anesthetized rats were transcardially perfused as previously described [21, 32]. Ultrathin sections were viewed with a Philips CM 100 electron microscope. At least five endplate regions were photographed from each muscle. Quantitative morphometry of the folding index (length of postsynaptic membrane/length of presynaptic membrane) was performed as previously described [34, 35]. For the analysis, the following number of animals was used per group: control saline (n = 3); EAMG saline (n = 4); EAMG 4w-Bz (n = 4); EAMG 8w-Bz (n = 4). Between 5 and 25 endplate regions were analyzed per animal.

Statistics

GraphPad Prism 4 was used to perform statistical analyses. Comparison between normally distributed values was performed using one- or two-way ANOVA, wherever appropriate. Bonferroni post hoc tests were used to compare groups to each other. A two-sided probability value of 0.05 or lower was considered significant. Values are expressed as means ± standard error of the mean (SEM) unless stated otherwise. Clinical scores were analyzed by the Chi square (χ^2) test for trend, and survival was analyzed using the log-rank test.

Results

Bortezomib reduces plasma cells in bone marrow

To investigate whether proteasome inhibition affects plasma cells *in vivo*, rats were injected with bortezomib or saline. Subcutaneous injections of bortezomib significantly reduced the proteasome activity in rat whole blood (Fig. 5.1). The effect of bortezomib on plasma cells from the bone marrow was analyzed by EM and by FACS. In the bone marrow of bortezomib-treated rats, plasma cells with altered morphology were frequently observed (Fig. 5.2), which was characterized by a vesicular appearance of the rough ER cisternae or pronounced dilatation of the rough ER. The percentage of plasma cells in bone marrow was markedly reduced in bortezomib-treated groups (Fig. 5.3*A*). Animals that received bortezomib only between 4 and 8 weeks after immunization showed a significant decrease in their percentage of bone marrow plasma cells (57% reduction in the 4w-Bz control group [$p < 0.05$] and 82% reduction in the 4w-Bz EAMG group [$p < 0.01$] compared with the corresponding saline-treated groups). Rats in the 8w-Bz EAMG group also showed a strong and significant depletion of bone marrow plasma cells ($p < 0.05$; 70% reduction compared to the saline-treated EAMG group). Very similar results were obtained by FACS analysis using intracellular staining of the Igκ light chain in bone marrow cells (Fig. 5.3*B*).

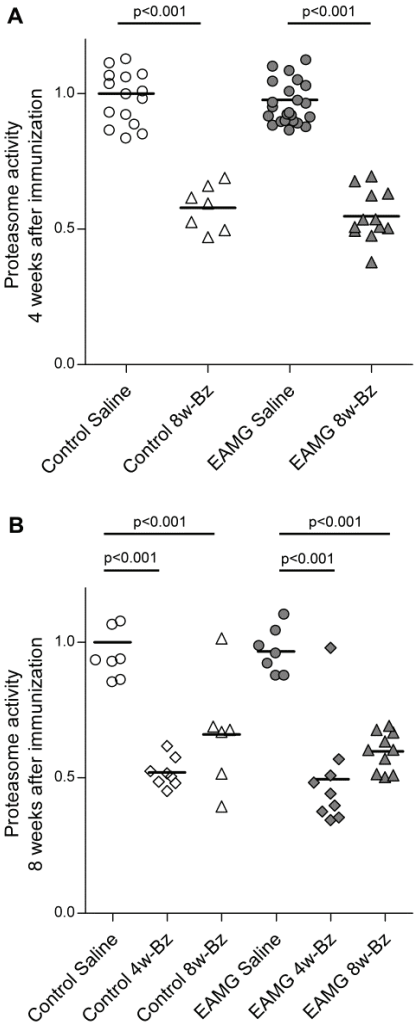

Figure 5.1. Proteasome activity in blood lysates. Samples were incubated with a labeled peptide substrate (LLVY-7-amino-4-methylcoumarin [AMC]) and the amount of cleaved fluorophore AMC was measured in a fluorometer. (*A*) Proteasome activities at 4 weeks after immunization were normalized using the average relative fluorescence units (RFU) value of the control saline group at week 4. (*B*) Proteasome activities at 8 weeks after immunization were normalized using the average RFU value of the control saline group at week 8. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.

Bortezomib affects the lymphoid organs

We investigated the overall effect of bortezomib treatment on the immune system by measuring the weight of thymus and spleen tissue and analyzing the proportions of B cells and T cell subsets in the thymus, spleen, blood and bone marrow by FACS (Table 5.2).

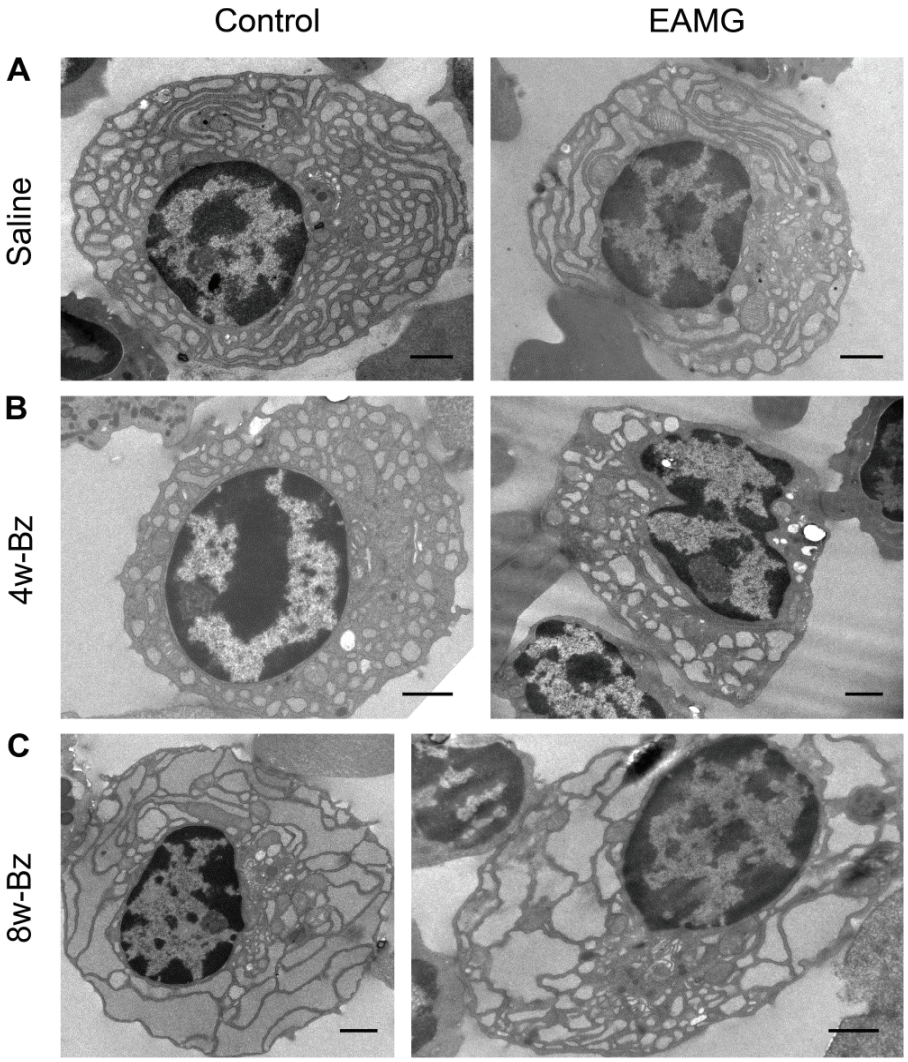

Figure 5.2. Electron micrographs of bone marrow plasma cells. After 4 weeks of bortezomib treatment, the rough endoplasmic reticulum (ER) cisternae have a vesicular appearance; after 8 weeks of treatment, pronounced dilatation of the rough ER is visible. Cells were stained with osmium tetroxide and contrasted with uranyl acetate and lead citrate. Scale bars, 1 µm.

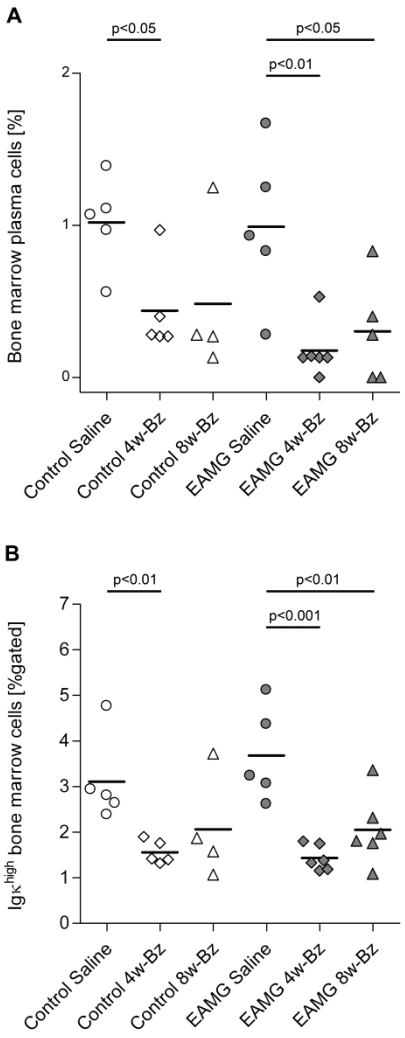

Figure 5.3. Analysis of plasma cells in the bone marrow 8 weeks after immunization. (*A*) Electron microscopical analysis. Bortezomib treatment decreases the number of plasma cells in the bone marrow. (*B*) Flow cytometric analyses of Igκ^{high} cells. Data are shown as percentages of cells with respect to gated living bone marrow cells. The proportion of Igκ^{high} cells was significantly lower in 4w-Bz and 8w-Bz groups compared with the corresponding saline-treated groups. Horizontal bars represent the mean percentage of each group. Two-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.

Bortezomib treatment significantly reduced the mean thymus weight, both in the 4w-Bz (46% reduction; $p < 0.01$) and 8w-Bz groups (50% reduction; $p < 0.01$), compared with the saline group (data not shown). In contrast, the mean spleen weight significantly increased by bortezomib treatment in both 4w-Bz (36% increase; $p < 0.05$) and 8w-Bz groups (32% increase; $p < 0.05$) compared to the corresponding saline-treated groups (data not shown). No significant difference was observed between control and EAMG animals.

To assess the effects of bortezomib treatment on the leukocyte viability, we measured the proportion of early apoptotic (annexin V^+/PI^-) and dead cells (annexin V^+/PI^+).

In the spleen, thymus, and bone marrow, a trend toward a higher proportion of early apoptotic and dead cells after bortezomib administration could be observed, but the differences did not reach statistical significance. However, the lymphocyte subpopulation in the bone marrow contained a significantly increased number of apoptotic or dead cells after bortezomib-treatment (data not shown).

Table 5.2. FACS analysis of lymphoid organs

	Tissue	Saline	4w-Bz	8w-Bz
Dead cells, Ann^+/PI^+	Thymus	11.25 ± 1.43	13.63 ± 1.17	14.19 ± 1.94
	Spleen	25.38 ± 2.34	28.00 ± 3.28	31.26 ± 4.25
	Bone Marrow	12.93 ± 0.86	14.57 ± 1.20	15.82 ± 1.16
	PBMC	5.72 ± 0.66	2.84 ± 0.17***	2.92 ± 0.18***
Early apoptotic cells, Ann^+/PI^-	Thymus	8.23 ± 0.96	12.11 ± 0.63	10.22 ± 1.60
	Spleen	17.02 ± 1.27	16.36 ± 1.62	15.80 ± 1.63
	Bone Marrow	12.40 ± 1.76	10.40 ± 1.19	13.08 ± 1.56
	PBMC	2.25 ± 0.18	1.23 ± 0.12***	1.91 ± 0.22
$CD45RA^+/Ig\kappa^+$	Thymus	0.48 ± 0.05	0.89 ± 0.12	0.63 ± 0.10
	Spleen	19.85 ± 0.36	15.85 ± 1.05*	16.13 ± 1.24*
	Bone Marrow	4.16 ± 0.16	2.56 ± 0.30**	2.44 ± 0.33***
	PBMC	5.64 ± 0.43	3.16 ± 0.26***	2.57 ± 0.18***
$CD3^+/CD4^+$ $CD8^-$	Thymus	7.72 ± 0.39	10.53 ± 0.65**	9.62 ± 0.61
	Spleen	39.44 ± 0.25	43.92 ± 1.53*	44.72 ± 0.71*
	Bone Marrow	1.27 ± 0.22	1.27 ± 0.31	1.49 ± 0.29
	PBMC	56.52 ± 0.68	63.21 ± 0.63***	62.18 ± 0.79***
$CD3^+/CD8^+$ $CD4^-$	Thymus	1.78 ± 0.11	3.49 ± 0.30***	2.80 ± 0.31*
	Spleen	12.89 ± 0.26	9.82 ± 0.39***	9.13 ± 0.63***
	Bone Marrow	1.67 ± 0.30	0.82 ± 0.08*	1.29 ± 0.20
	PBMC	19.48 ± 0.40	20.91 ± 0.47	20.85 ± 0.43
$CD4^+/CD8^+$	Thymus	83.57 ± 1.24	77.41 ± 1.87*	78.99 ± 1.38
	Spleen	1.33 ± 0.06	1.27 ± 0.07	1.46 ± 0.07
	Bone Marrow	1.60 ± 0.39	1.03 ± 0.11	0.99 ± 0.12
	PBMC	1.39 ± 0.12	1.62 ± 0.13	2.16 ± 0.19**

Using flow cytometry, the effects of bortezomib treatment on apoptosis and on lymphocytes were measured in the thymus, spleen, bone marrow, and peripheral blood. Results are shown as average percentages ± SEM. One-way ANOVA analysis and Bonferroni post hoc testing were utilized for statistical analysis. For analyzing the effect of bortezomib treatment, the data of control and EAMG groups were combined since no significant differences were observed between them in any of the parameters studied.
*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Ann, annexin V.

In the peripheral blood, unexpectedly, bortezomib treatment significantly decreased the proportion of dead cells in PBMCs in the 4w-Bz and 8w-Bz groups compared

to the saline groups. The proportion of early apoptotic cells in PBMCs was significantly lower in the 4w-Bz groups ($p < 0.001$) but not in the 8w-Bz groups.

In the spleen, the bone marrow, and the blood, the proportion of $CD45RA^+/Ig\kappa^+$ B cells was significantly decreased after bortezomib treatment (Table 5.2), both in the 4w-Bz and the 8w-Bz groups.

After treatment with bortezomib, the proportion of immature $CD4^+/CD8^+$ cells in the thymus was decreased whereas the proportion of $CD3^+/CD4^+/CD8^-$ T helper cells and cytotoxic $CD3^+/CD8^+/CD4^-$ T cells were increased.

In the blood and the spleen, $CD3^+/CD4^+/CD8^-$ cells were significantly increased; the proportion of $CD3^+/CD8^+/CD4^-$ cells was significantly decreased in the spleen and bone marrow of bortezomib-treated rats.

In general, the two bortezomib treatment regimes led to similar changes of the rat immune system, with the exception of the proportion of apoptotic cells, as aforementioned.

Total IgG concentration is decreased by bortezomib

The effect of bortezomib on total IgG content in plasma samples was measured by ELISA (Fig. 5.4*A*). In comparison with the saline-treated group, total IgG at week 8 was significantly reduced in both the 4w-Bz and the 8w-Bz group ($p < 0.001$). Importantly, this immunosuppressive effect of bortezomib was observed in both control and EAMG animals, although IgG reduction was more pronounced in 8w-Bz EAMG animals than in the 4w-Bz EAMG rats ($p < 0.05$). A slightly but significantly higher concentration of IgG was observed in all EAMG groups in comparison with the corresponding control groups. Compared to the saline-treated groups, a highly significant reduction in IgG concentrations ($p < 0.001$) was already achieved after 4 weeks of bortezomib treatment in the 8w-Bz group (data not shown).

Bortezomib reduces autoantibody titers in EAMG

The plasma concentration of autoantibodies to the rat AChR was measured by RIA. Autoantibodies were detectable 4 weeks after immunization in all EAMG animals and reached very high levels after 8 weeks (Fig. 5.4*B*). The variability of autoantibody levels

between animals is typical for the EAMG model, but it should be borne in mind that already a titer of 1 nM of autoantibodies is sufficient to cause substantial damage to the NMJ [33]. As expected, no anti-AChR antibody titers could be detected in sham/CFA-immunized animals (control group; data not shown). On the other hand, animals that received bortezomib injections from the moment of immunization showed a significantly lower production of autoantibodies (p < 0.01; corresponding to a 72% reduction of average autoantibody titer) compared with the saline treated EAMG group 8 weeks after immunization (Fig. 5.4*C*). Interestingly, rats that received bortezomib 4 weeks after immunization had an autoantibody production profile similar to that observed in the 8w-Bz group. After injection of bortezomib, the production of anti-AChR antibodies was reduced significantly compared with saline-treated animals (corresponding to a 60% reduction; p < 0.01). This demonstrated that bortezomib effectively diminished antibody production not only when administrated at the moment of immunization but also once the immune response was already ongoing.

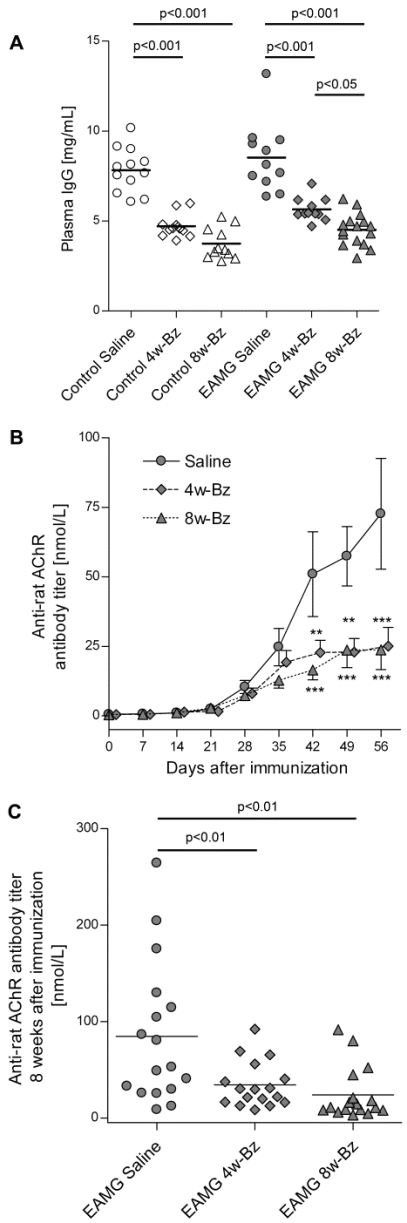

Figure 5.4. Total IgG and autoantibody titers in plasma. (*A*) Plasma IgG titers 8 weeks after immunization. Bortezomib significantly reduced plasma IgG levels. Two-way ANOVA and Bonferroni post hoc testing were used for statistical analyses. (*B*) Average anti-rat AChR-titer; error bars correspond to the SEM. Stars indicate significant differences compared to the saline-treated groups. **p < 0.01; ***p < 0.001 compared with the saline-treated groups. Autoantibody titers of the 4w-Bz and the 8w-Bz groups were not significantly different from each other at any time point. (*C*) Anti-rat AChR titers 8 weeks after immunization with tAChR. One-way

ANOVA and Bonferroni post hoc testing were used forstatistical analyses.

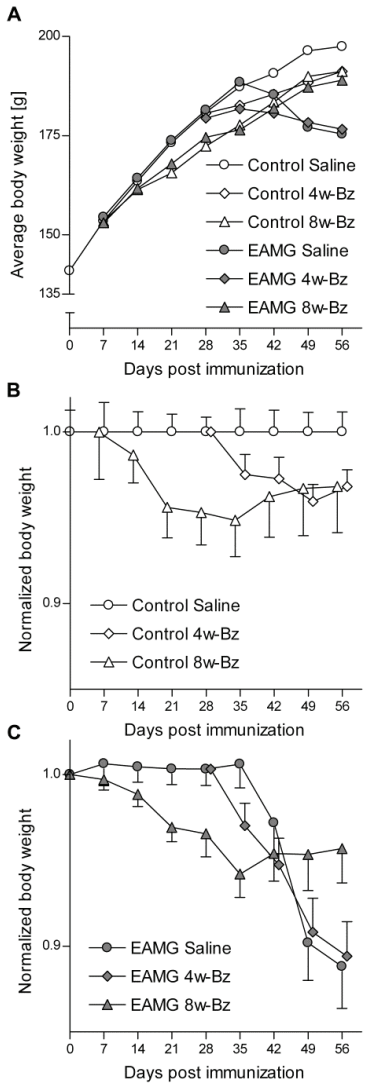

Figure 5.5. Average rat weights. (*A*) Body weights of bortezomib-treated animals increased slower compared with saline-treated animals. Weight loss occurred frequently in EAMG animals. (*B, C*) Normalized weights were calculated using the average weight of the saline–treated control group. Error bars correspond to the SEM.

Bortezomib ameliorates clinical conditions in EAMG

To assess the effect of bortezomib on the overall condition of experimental animals, we weighed them and scored their clinical status on a weekly basis. During the first 5 weeks of treatment, control animals that had received bortezomib from the moment of immunization (8w-Bz group) showed a slower increase in their total body weight compared to saline-treated control animals (Figure 5.5*A* and *B*; $p < 0.001$). After 5 weeks, the growth of these animals normalized again. A similar reduction of growth was observed 4 weeks later in the 4w-Bz control group. At the end of the experiment there were no significant differences between the average weights of the 4w-Bz and the 8w-Bz control groups. As expected, control animals did not present muscle weakness or any other clinical sign of EAMG.

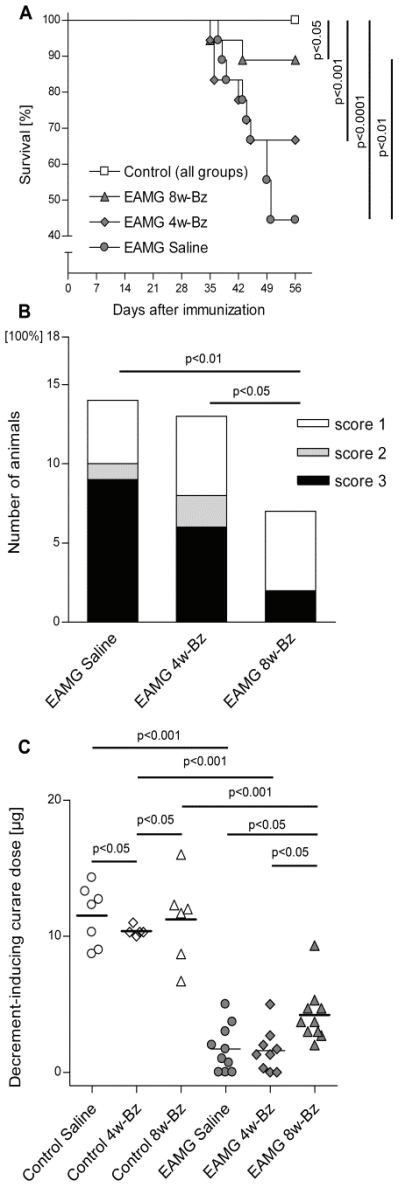

Figure 5.6. MG symptoms and muscle function. (*A*) Survival. (*B*) Clinical scores of muscle weakness. Each group contained 18 animals (indicated with 100%). (*C*) EMG after curare infusion. The curare dose that induces at least 10% decrement of the CMAP was used as a measure of the neuromuscular safety factor.

Animals immunized with *Torpedo* AChR developed clinical symptoms of EAMG starting 5 weeks after immunization, when antibody titers reached considerable levels. Frequently, weight loss preceded the observation of other myasthenic symptoms in EAMG animals, indicating weakness of bulbar muscles and difficulties in chewing and swallowing.

The disease in some of these animals progressed rapidly to score 3 within two days and they had to be sacrificed (Fig. 5.6*A*). By the end of the experiment 50% of animals (9 of 18) treated with saline solution reached a clinical score of 3 or lost >20% body weight and had to be sacrificed for ethical reasons. This percentage was reduced to 33% (6 of 18) in the 4w-Bz group and to 11% (2 of 18) in the 8w-Bz group (Fig. 5.6*B*). The survival rate of the 8w-Bz EAMG group was significantly higher compared with the saline-treated EAMG group ($p < 0.01$; Fig. 5.6*A*).

From the AChR-immunized animals, 78% developed muscle weakness in the saline-treated EAMG group, 72% in the 4w-Bz EAMG group, and 39% in the 8w-Bz EAMG group. The clinical score of the 8w-Bz EAMG group was significantly lower compared with the 4w-Bz group ($p < 0.05$) and the saline-treated EAMG group ($p < 0.01$; Fig. 5.6*B*).

Despite the reduced amount of autoantibodies, the muscle weakness and the survival rate of the 4w-Bz EAMG group were not significantly different from the saline-treated EAMG group. The onset of weight loss in the 4w-Bz EAMG group even occurred somewhat earlier compared to the saline-treated EAMG group (Fig. 5.5*C*), indicating that weight loss was partly caused by the (side) effects of bortezomib.

We evaluated the safety factor of neuromuscular transmission, which is a function of the postsynaptic density of AChRs, by performing EMG studies in the presence of the AChR-blocking agent (+)-tubocurarine (curare). The amount of curare needed to induce a decrement in CMAP is related to the safety factor of neuromuscular transmission. Bortezomib had slight effects on the curare sensitivity of the NMJ in control animals in the 4w-Bz group (Fig. 5.6*C*). The neuromuscular transmission was significantly impaired in all EAMG groups compared with the corresponding control groups. In the 8w-Bz EAMG group, neuromuscular transmission was significantly improved compared with the saline-treated and the 4w-Bz EAMG groups ($p < 0.05$).

Ultrastructural analysis of the NMJ revealed damage of the postsynaptic membrane morphology, with degenerating or absent secondary clefts in EAMG animals (Fig. 5.7). The damage in endplates of animals with low titers in the 8w-Bz group (Fig. 5.7*F*) was less severe compared with animals with higher titers (Fig. 5.7*D, E*, *G*). Quantitative morphometric analysis of the synapse ultrastructure was performed to measure the loss of postsynaptic folds (Fig. 5.8). The folding index was significantly reduced in the saline-treated EAMG animals compared with the saline-treated control animals (a reduction of 55%, $p < 0.001$). In the 8w-Bz EAMG animals only a 20% reduction of the folding index compared with saline-treated control animals was observed, and a considerable proportion of the endplates had a relatively high folding index. Compared to the saline-treated EAMG group, the folding index in the 8w-Bz group was significantly higher ($p < 0.001$). However, this treatment effect was not observed in the 4w-Bz group. In conclusion, bortezomib could not improve synaptic ultrastructure if treatment was started 4 weeks after immunization, but it efficiently prevented damage of the postsynaptic membrane when administrated for 8 weeks starting directly after immunization.

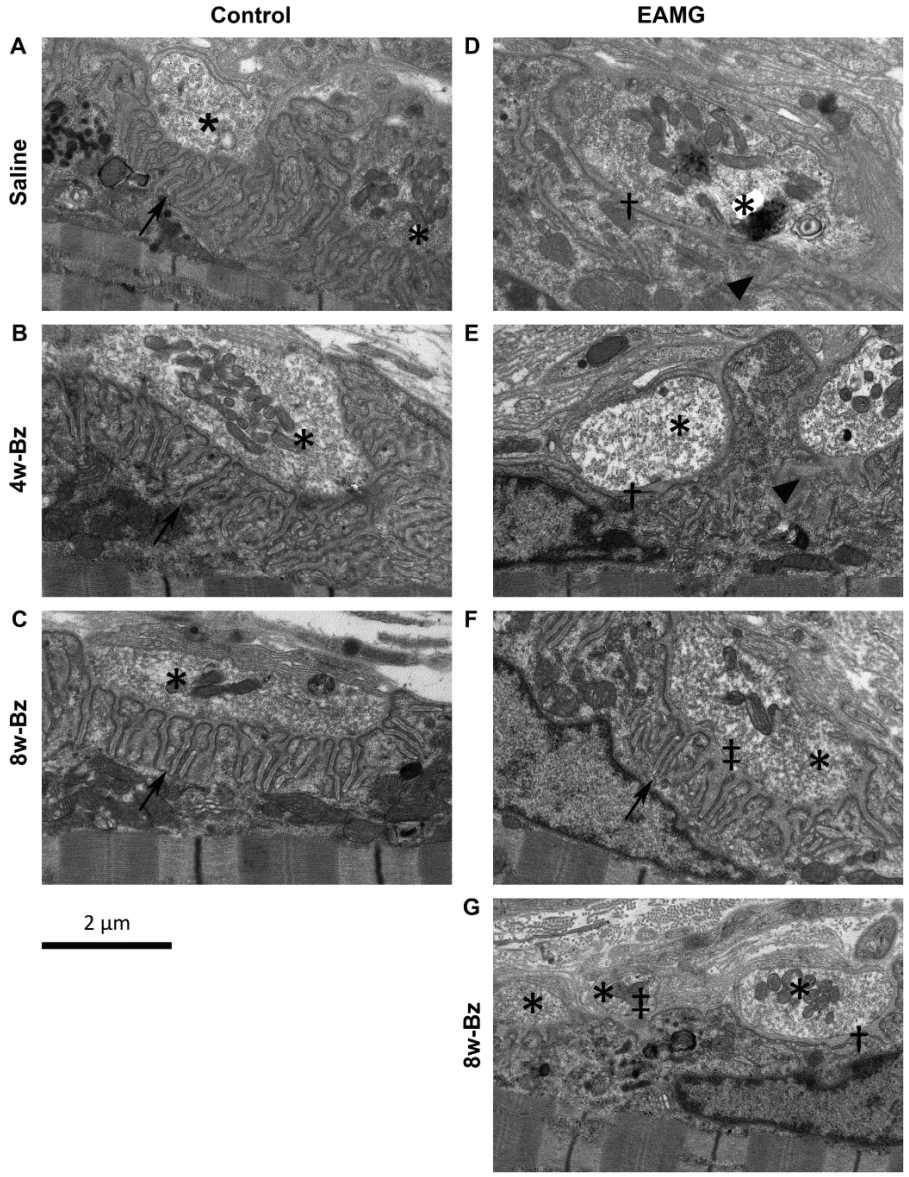

Figure 5.7. Electron micrographs of synaptic boutons of the NMJ. Nerve terminals are indicated by asterisks. In control animals (*A-C*), the postsynaptic membrane contains secondary clefts (postsynaptic folds), which are indicated by arrows. In EAMG animals (*D-G*), pathologic changes of the postsynaptic membrane are indicated: degenerating folds (arrowhead), simplified and without folds (dagger), and widening of the primary and secondary synaptic clefts (double dagger). The postsynaptic damage of an animal with an anti-AChR titer of 3 nM (*F*) was mild compared to an animal with a titer of 20 nM (*G*). The muscle tissue was stained with osmium tetroxide and contrasted with uranyl acetate and lead citrate.

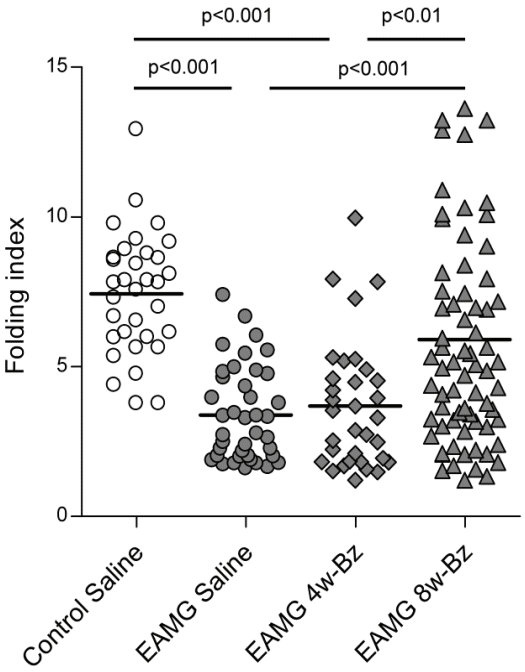

Figure 5.8. Analysis of folding index (length of postsynaptic membrane/length of presynaptic membrane) by quantitative morphometry of electron micrographs. Each point represents one endplate region with one or two synaptic boutons as shown in Fig. 5.7. Due to loss of postsynaptic folds, the folding index is significantly reduced in EAMG saline animals compared with control saline animals. In the EAMG 8w-Bz, the reduction of postsynaptic folding was prevented by bortezomib. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.

Discussion

In this study we analyzed the effect of proteasome inhibition on the autoimmune response in the EAMG model for MG. Bortezomib reduced the amount of plasma cells, leading to a significant reduction of total serum IgG and autoantibody levels and caused an amelioration of myasthenic symptoms compared with a saline-treated EAMG group. Both the 4- and 8-week bortezomib treatment regimes (4w-Bz and 8w-Bz) were generally well tolerated, but some side effects were noticed, that are discussed below. There were no significant differences in autoantibody production between the 4w-Bz and the 8w-Bz group. Because bortezomib in the 4w-Bz group was only administered starting 4 weeks

after immunization with autoantigen, this suggests that mainly the late (effector) phase of the autoimmune response is affected by proteasome inhibition.

In the EAMG model, the production of antibodies against the muscle AChR is induced by immunization with tAChR. EAMG in Lewis rats is characterized by chronic muscle weakness starting 5 weeks after immunization. A transient acute phase of muscle weakness starting 1 week after immunization is seen in EAMG models using CFA with *Bordetella pertussis* [20]. In the immunization protocol used in this study with CFA containing *M. tuberculosis*, the acute phase of muscle weakness does not occur [20]. Therefore, we could investigate both a therapeutic as well as a preventive treatment regimen. Bortezomib efficiently reduced the production of autoantibodies and also ameliorated MG symptoms in the 8w-Bz group. The average levels of anti-AChR autoantibodies in this study were high, comparable to a previous study using the same protocol with 20 µg tAChR [36, 37] and much higher than another study using 10 µg tAChR [33], ranging between 3 and 80 nM even in the 8w-Bz groups. Because 1 nM serum anti-muscle AChR-antibodies can already reduce the amount of total muscle AChR by 50% in rats [33], the limited improvement of muscle strength in this study is understandable. It is therefore conceivable that if we could have used EAMG animals with a lower titer, the effects of bortezomib would have been more pronounced. Nevertheless, the resistance against curare, and thus the amount of functional AChR at the NMJ and the folding index of the postsynaptic membrane, was significantly increased by bortezomib in the 8w-Bz treatment group, resulting in higher survival rates. In human MG patients, an autoantibody titer reduction of 50% after plasma exchange is generally sufficient to achieve clinical remission [38, 39], and therefore in this respect the bortezomib-induced reduction of autoantibody production by > 65% within 4 weeks is therapeutically promising.

However, despite the reduced autoantibody levels, the 4w-Bz treatment did not result in a significant improvement of health in EAMG animals, in contrast to the 8w-Bz treatment, where bortezomib caused amelioration in the condition of the animals. Two side effects of bortezomib might have influenced this result. First, bortezomib negatively affected body weight during the first 4 weeks of administration in control animals and

presumably also in EAMG animals. In the 4w-Bz EAMG group, this coincided with the weight loss as a result of muscle weakness and the resulting problems with eating and drinking. Because a 20% weight loss was chosen as a criterion to sacrifice animals for ethical reasons, the bortezomib treatment could have reduced survival time to some extent. Second, we observed a mild impairment of neuromuscular transmission in the 4w-Bz Control group (but not in the 8w-Bz control group) in comparison with the saline-treated control group. This effect possibly indicates transient nerve damage similar to the bortezomib-induced polyneuropathy [26, 40]. Arguably, the weight loss in control animals was caused by an effect of proteasome inhibition on the gastrointestinal tract, which has been seen in patients [41]. However, our study was not designed for investigating the side effects of bortezomib, and therefore we cannot conclusively attribute the effect on weight to any particular side effect. In the 8w-Bz Control group, weight gain, neuromuscular transmission and apoptosis were normalized 8 weeks after immunization, suggesting that by that time compensatory mechanisms limited these adverse effects of bortezomib.

Despite comparable autoantibody titers, the ultrastructural postsynaptic damage of the 4w-Bz EAMG group was significantly higher compared with the 8w-Bz EAMG group. Because the repair of the postsynaptic membrane takes at least 10 days [42], it seems possible that the observed loss of postsynaptic folding in the 4w-Bz group is the result of earlier damage, in particular in the period between 4 and 7 weeks after immunization, when titers were higher compared with the 8w-Bz EAMG group.

Apart from the intended killing of plasma cells, bortezomib affected the immune system in a more general fashion. In particular, the thymus was affected by bortezomib; an effect that might not be harmful, since thymectomy is a frequently used treatment in MG, albeit with unproven efficacy so far. In this respect it is relevant that in human thymocyte cultures from thymectomized MG patients we observed that bortezomib induced cell death and reduction of autoantibody production (A. Gomez, K. Vrolix, and M. Losen, unpublished observations). Similar to a previous study in mice [43], we found in our rat model that bortezomib mainly affects immature thymocytes. In the spleen, bone marrow, and the blood of rats, the proportion of $CD45RA^+/Ig\kappa^+$ B cells was significantly decreased, whereas the proportion of $CD3^+$ T cells of total lymphocytes was increased.

There was a trend for increased proportion of dead and apoptotic cells in the thymus, bone marrow, and spleen of bortezomib-treated animals. In contrast, bortezomib induced a significant reduction of apoptotic cells in the blood. Because the PBMCs only represent a minor proportion of leukocytes, this effect could be attributed to migration of cells into other lymphoid organs, for example, the spleen, which was significantly enlarged in bortezomib-treated rats.

In the past the EAMG model has been instrumental for testing the efficacy of new therapies that are now used for the treatment of MG [reviewed by 44], for example, cyclophosphamide [45], pixantrone [46], linomide [47], azathioprine, and hydrocortisone [48]. Also, mycophenolate mofetil efficiently reduces autoantibodies in a rat model of EAMG [33]. This study now indicates that bortezomib might be useful to complement these drugs for the treatment of MG since it can additionally target plasma cells that produce autoantibodies. Because of the observed plasma cell reduction in rats, it is reasonable to presume that a course of bortezomib treatment might well eliminate short- and long-lived plasma cells also in humans and therefore induce a long-lasting treatment response in antibody-mediated autoimmune diseases.

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Conflicts of interest: R.A.M. and R.E.V. have applied for a patent on the use of proteasome inhibitors for plasma cell depletion. The other authors have no financial

conflicts of interest.

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Chapter 6

Proteasome inhibition with bortezomib depletes plasma cells and specific autoantibody production in primary thymic cell cultures from early-onset myasthenia gravis patients

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Abstract

Bortezomib is a potent inhibitor of proteasomes currently used to eliminate malignant plasma cells in multiple myeloma patients. It is also effective in depleting both alloreactive plasma cells in acute antibody-mediated transplant rejection and their autoreactive counterparts in animal models of lupus and myasthenia gravis (MG).

In this study, we demonstrate that bortezomib killed long-lived plasma cells in cultured thymus cells from early-onset MG patients – even at 10 – 250 nM – and halted their spontaneous production not only of autoantibodies against the acetylcholine receptor but also of total IgG. Bortezomib-treated plasma cells showed ultrastructural changes characteristic of endoplasmic reticulum stress after 8 hours, and were no longer detectable at 24 hours. Bortezomib therefore appears promising for treating MG and possibly other antibody-mediated autoimmune or allergic disorders, especially if given in short courses at modest doses before the standard immunosuppressive drugs have taken effect.

Introduction

Myasthenia gravis (MG) with antibodies against the muscle acetylcholine receptor (AChR) is one of the best understood of the numerous autoimmune neurological diseases now recognized [1]. It is generally agreed that the patients' autoantibodies are pathogenic, as they decrease AChR numbers by antigenic modulation and complement-mediated damage [2, 3]. Patients with early-onset MG (EOMG; before age 45) are an unusually well defined subgroup, with strong female and HLA-B8 biases [4], and characteristic lymph node-like infiltrates in the thymic medulla [5-7].

Treatment of MG relies primarily on glucocorticoids, often combined with broad-spectrum immunosuppressants such as azathioprine or mycophenolate mofetil (MMF), or rituximab (anti-CD20 mAb) [8]. However, their efficacy and side-effects vary greatly between patients and they reduce autoantibody titers and restore muscle strength only after delays as long as 4 - 15 months [9, 10]. In addition, drug-resistant AChR-MG patients treated with rituximab showed no reduction in either anti-AChR antibody titers or IgG levels, despite complete elimination of circulating B-cells [10]. In such patients, long-lived plasma cells, which are CD20 negative, are likely to be the main producers of the autoantibodies. Moreover, they are probably responsible for the delayed responses of most MG patients to immunosuppressants, which mainly act by preventing generation of new plasma cells from B-cells and impairing the activation and proliferation of T-helper cells [11-13].

Plasma cells are terminally differentiated high-rate antibody-secreting cells [>10,000 molecules per cell per second [14, 15]] which do not divide; among the B-cell lineage, they are uniquely radio-resistant. Whereas some are short-lived, others persist for many months (or even years) [16] in special survival niches in bone marrow [17] and lymphoid tissues [18]. They are the main producers of circulating IgG, and are clearly major contributors in chronic antibody-mediated autoimmune diseases. Their resistance to both standard immunosuppressants and rituximab, therefore, necessitates a different pharmacological approach. Proteasome inhibitors are a promising new class of drugs for targeting plasma cells [reviewed in: 18, 19, 20]. Bortezomib was the first clinically

approved proteasome inhibitor and is used for treating multiple myelomas (MM), neoplasms of plasma cells. In addition, bortezomib is now used to prevent acute antibody-mediated rejection of solid organ transplants [21]. It is also showing promise in antibody-mediated autoimmune diseases such as systemic lupus erythematosus (SLE) and thrombotic thrombocytopenic purpura (TTP) [18, 22]. In autoimmune animal models of SLE, ANCA-induced glomerulonephritis and MG, it depleted both plasma cells and autoantibodies [23-25].

Partly because of their high rate of protein synthesis and dependence on protective unfolded protein responses, MM cells are very susceptible to proteasome inhibitors [26]. These rapidly induce apoptosis by activating the terminal unfolded protein response [27] and inhibiting the transcription factor NF-κB [28]. Proteasome inhibition has similar effects on non-neoplastic plasma cells *in vivo* [24].

Non-neoplastic plasma cells may also be susceptible to other anti-myeloma drugs, for example, the thalidomide derivative lenalidomide, which is frequently combined with dexamethasone in non-pregnant MM patients, and appears relatively safe. Lenalidomide inhibits the proliferation of several MM cell lines and disrupts the stromal support in their survival niches [29]. Since it reduces IgM and IgG responses to pokeweed mitogen (PWM)-stimulated B-cells [30], it must affect earlier B-lineage cells too.

In most EOMG patients, the thymic infiltrates include numerous germinal centers [5], many of them AChR-specific, and autoreactive T- and B-cells along with terminal plasma cells [31]. In primary cultures of cells from EOMG but not control thymi, autoreactive plasma cells spontaneously secrete AChR autoantibodies, with titers and fine specificities very similar to those in the patients' sera [31, 32]. They do so for several weeks (at least) – even after irradiation [32], implying that many of them are long-lived. The resilience of these plasma cells contrasts strikingly with the majority of thymic subsets, e.g. immature thymocytes and T-cells, which have a very high turnover *in vivo* [33, 34] and die rapidly in culture [35].

Thymectomy is part of standard management of EOMG in many centers. Thus the tissue removed is an almost uniquely accessible source of long-lived human autoimmune plasma cells. Here, we have for the first time tested the hypothesized potential of

bortezomib and lenalidomide to target such plasma cells.

Patients, Materials and Methods

Patients

The MG patients (MG 1 - 3) were thymectomized 6 - 30 months after MG onset in London (UK), and were all young females with typical EOMG (onset-ages, 16, 20 and 24 years) but no steroid pre-treatment. They were recruited with informed consent and Ethics Committee approval, and selected only because of recent MG-onset, high serum anti-AChR titers (180, 270 and 1,000 nM), correspondingly high productivity of these antibodies by their thymic cells in culture [32], and availability of irradiated cells. To assess total cell survival after administration of drugs, we used thymus cells from a fourth patient (MG onset-age 36 years, i.e. 13 months before thymectomy; anti-AChR titer 24 nM).

Cell culture and experimental design

Thymic cells were cultured as described [36]. Briefly, fresh thymic tissue was dispersed enzymatically, washed (and some aliquots irradiated with 1,250 rads from a ^{60}Co source) and cryo-stored within a few hours; subsequently, they were thawed carefully, and cultured at 6x 10^5 – 9x 10^5 cells per well in 96 well round-bottomed plates, without added stimulants in 200 µL of RPMI medium containing 15% fetal bovine serum (Bodinco, the Netherlands), 50 U/mL penicillin, 50 U/mL streptomycin and 1 mM sodium pyruvate, at 37°C in humidified air with 5% CO_2. Every 2 - 3 days, we removed (and stored) 90 µL of supernatant from each well, and replaced it with 100 µL of fresh medium ± any test drugs.

We dissolved lyophilized bortezomib (Velcade, Janssen-Cilag B.V., Belgium) in sterile saline, dexamethasone (D4902; Sigma-Aldrich) in absolute ethanol, and lenalidomide (Santa Cruz Biotechnology; sc-218656) in dimethyl sulfoxide.

Immunofluorescence staining and enumeration of plasma cells

Cultured cells (5x 10^4 - 5x 10^5) were cytocentrifuged onto poly-L-lysine-coated slides (for 5 minutes at ~120 g; Cyto-Tek centrifuge model 4332; Sakura Finetek, Japan). Slides were air-dried for 1 hour at 22°C and then fixed in 4% paraformaldehyde at 4°C for 10 minutes.

Subsequently, they were blocked with 2% bovine serum albumin in PBS and incubated with Hoechst 33342 solution (2 µg/mL, Cat. B2261; Sigma-Aldrich) to stain DNA. Plasma cells were stained with mouse anti-human CD138 mAb (1:250, Clone MI15, Dako), donkey anti-mouse IgG Alexa 594 (1:300, A21203; Molecular Probes-Invitrogen) and goat anti-human IgG Alexa 488 (1:500, A11013; Molecular Probes-Invitrogen). Slides were mounted in 80% glycerol-TBS and stored at 4°C in the dark. All washing and incubation steps were performed with TBS-Triton X-100 (0.03%). We counted the plasma cells from each well, in a blinded fashion, on a fluorescence microscope (Olympus BX51), identifying them by their distinctive size, shape (extensive cytoplasm and eccentric nuclei), and positive staining for IgG and/or CD138. Results are expressed as the proportion of the initially plated plasma cells that were finally detected in each well (% survival PC).

Autoantibody and total IgG assays

In a standard radio-immunoprecipitation assay, we incubated 20 µL of supernatant overnight at 4°C with 12.5 µL of TE671 human rhabdomyosarcoma cell membrane-extract (containing approximately 3 fmol of human AChR). The AChR was labeled with excess ^{125}I-α-bungarotoxin (^{125}I-α-BT, NEX126, 3.4 TBq/mmol; PerkinElmer), and normal human serum was used as carrier. Any immune complexes were precipitated by addition of 150 µL of goat anti-human IgG and incubation for 4 hours at 4°C. A standard curve was made by serial dilutions of the anti-AChR mAb 637 [37] and processed in parallel to the samples. Results are expressed as nanomoles of α-BT binding sites/L culture medium/day.

We measured total IgG in the culture supernatants with a standard sandwich ELISA as described [23], capturing with goat F(ab')$_2$ anti-human IgG-Fcγ (109-006-008; Jackson Immuno-Research; diluted 1:200) and detecting with horseradish peroxidase - conjugated goat F(ab')$_2$ anti-human IgG-Fcγ (109-036-008; Jackson Immuno-Research; diluted 1:20,000). Results were expressed as ng of total IgG secreted per mL of culture medium/day.

The newly synthesized antibody against the AChR and total IgG was quantitated as its present concentration (in 200 µL) minus the concentration in the previous sample (in 100 µL) / time interval.

Staining for surface B- and T-cell markers, and membrane integrity

Cells were incubated for 30 minutes at 4°C in FACS buffer (2% fetal calf serum and 0.1% sodium azide in PBS) with FITC mouse anti-human CD3 (Cat. 555339; BD Biosciences) and PE mouse anti-human CD19 (Cat. 555413, BD Biosciences), all diluted 1:100. The samples were washed twice, kept at 4°C in the dark, and analyzed within 2 hours. Dead cells were identified by propidium iodide (PI) counter-staining (Apoptosis detection kit; BD Pharmingen).

We used a FACSCalibur plus CellQuest Software (BD Biosciences) for data acquisition and analyzed results using the Flowing Software (version 2.5.0; Turku Center for Biotechnology, Finland). Samples were gated to exclude cell debris and aggregates, and PI-positive cells.

Electron microscopy

Cultured thymic cells were collected, pelleted (7 min, 220 *g*, 4°C) and fixed with 3% glutaraldehyde + 1.4% sucrose buffered in 0.09 M KH_2PO_4 at pH 7.4. They were then washed in 0.09 M KH_2PO_4 buffer with 7.5% sucrose and transferred to a 1% OsO_4 + 1.5% ferrocyanide solution buffered with veronal at pH 7.4 for subsequent immersion fixation for 1 hour at 4°C. After washing in veronal buffer with 7% sucrose at pH 7.4, dehydration was carried out rapidly in graded ethanol series. Samples were then incubated overnight in propylene oxide and Epon (1:1), and subsequently embedded in Epon. Serial 80 nm sections were stained with uranyl acetate, lead citrate, and coded. We used a Philips CM100 electron microscope to count plasma cells and examine their ultrastructure in five representative sections for each sample.

Statistics

GraphPad Prism 4 was used for statistical analyses. We compared normally distributed values using 1- or 2-way ANOVA analyses, and Bonferroni post-hoc tests. A two-sided probability value of 0.05 or lower was considered significant. Values are expressed as means ± standard error of the mean (SEM) unless stated otherwise. We used Spearman (non-parametric) correlation coefficients (ρ).

Results

Culturing EOMG thymic cells

Plasma cells were identified by their characteristic ultra-structural morphology (Fig. 6.1), intense internal IgG staining and surface CD138 expression (Fig. 6.2*A*). They were frequently found in clumps of 3-5 cells (or sometimes more), in close contact with extracellular matrix and other cell types (Fig. 6.2*A*), as in their survival niches in the spleen or bone marrow [38].

In the thymus, there is normally a high rate of cell death *in vivo* [33, 34]. As expected, it was also substantial in our suspension cultures of frozen/thawed thymic cells; ~20% of the input cells remained viable on day 14, and even fewer in irradiated samples (~8%). To maximize plasma cell recovery/activity, we used cryo-stored cells that had been dispersed with dispase and collagenase [7, 36]. They appeared highly dependent on cell concentration and on adherent 'feeder' fibroblasts and macrophages [36]. Although microenvironments were probably not optimal in the present cultures, spontaneous autoantibody production was nonetheless relatively consistent in quadruplicate wells (see Figs. 6.2, 6.3). Remarkably, it persisted for at least 2 weeks, even after irradiation (Fig. 6.3), when only occasional viable macrophages and fibroblasts could still be seen (not shown).

Bortezomib rapidly induces apoptosis in plasma cells from EOMG thymi

We cultured thawed EOMG thymic cells for 3 days before adding bortezomib; the standard concentration of 2.5 μM was based on previous *in vitro* experiments on human plasma cells [39] and the peak concentration measured in MM patients [40].

In all control samples, plasma cell ultrastructure appeared normal, with elaborate endoplasmic reticulum (ER), a well-defined Golgi complex and dense regions of (nuclear) heterochromatin in a typical "cart-wheel" distribution (Fig. 6.1*A*). They still appeared normal at 2 and 4 hours after addition of bortezomib (at 2.5 μM; Fig. 6.1*B*). However, after 8 hours, most surviving plasma cells showed signs of apoptosis (Fig. 6.1*C*), including condensation of chromatin and distension of the ER [41]. After 24 hours, they were no

longer detectable in the bortezomib-treated cultures. Results were very similar with bortezomib at 0.25 µM (not shown).

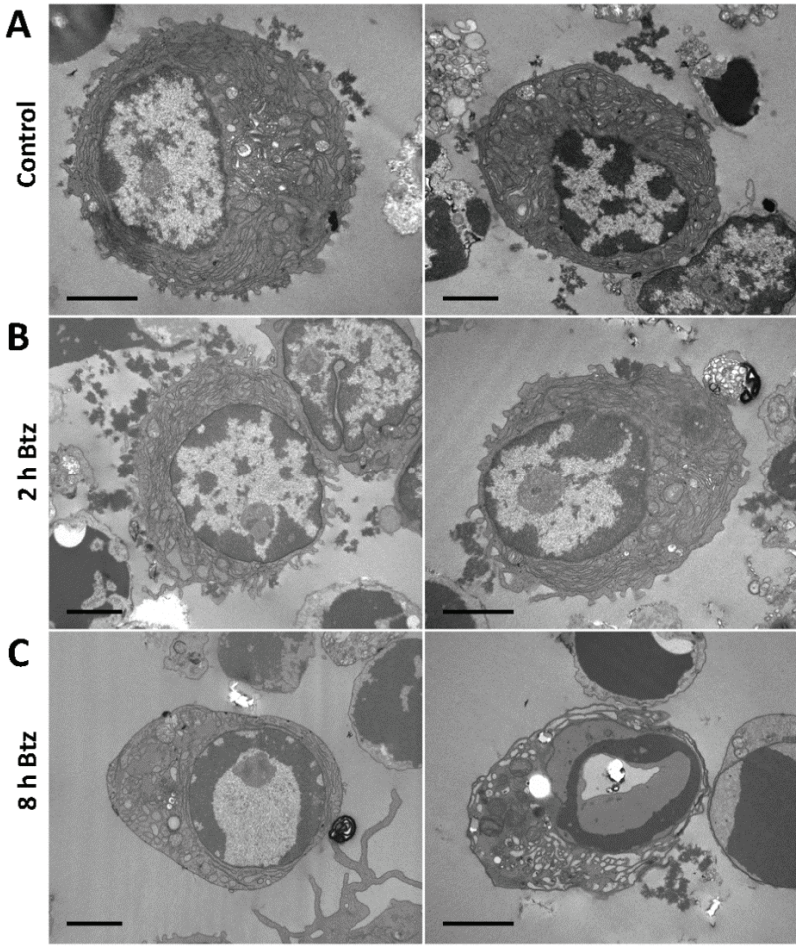

Figure 6.1. Ultrastructural changes in plasma cells incubated with bortezomib. (A) Normal plasma cells in control samples. Note the typical morphology, with an elaborate endoplasmic reticulum (ER) and eccentric nuclei (with a cart-wheel configuration of the heterochromatin). (B) Most plasma cells also appeared normal after 2 hours of treatment with bortezomib. (C) After 8 hours of treatment with 2.5 µM bortezomib, most plasma cells appeared apoptotic, with heterochromatin condensed around the perimeter of the nucleus and distension of the ER lumen. 24 hours after treatment with bortezomib, no plasma cells could be detected. Coded cell samples were post-fixed with osmium tetroxide and counter-stained with uranyl acetate and lead citrate. Scale bars are 2 µm.

Bortezomib eliminates plasma cells in cultured EOMG thymic cells

To focus on long-lived plasma cells, we next added bortezomib, lenalidomide or

dexamethasone on days 7 and 11 of culture, and counted surviving plasma cells on day 14. Lenalidomide was used at 10 µM, based on previous *in vitro* studies [42-44] and peak levels in MM patients [45]. Dexamethasone was tested at 10 nM, a level known to inhibit lymphocyte proliferation in susceptible humans [46, 47].

Three days after a second dose of bortezomib, plasma cells were almost undetectable (p<0.001; Fig. 6.2*B*). Interestingly, their numbers were not changed by lenalidomide, and only modestly reduced by dexamethasone (p<0.05). In a separate experiment, we confirmed that 10 µM lenalidomide suppressed IgG production by PWM-stimulated PBMCs (not shown), as previously reported [30].

Proteasome inhibition halts spontaneous secretion of total IgG and AChR autoantibodies

We could already detect significant production of both anti-AChR antibody and total IgG at 48 hours of culture onwards; both increased further from days 9 - 14 in the control and lenalidomide cultures (Fig. 6.2*C, E*). In striking contrast, production of both anti-AChR antibody and total IgG declined sharply after the first dose of bortezomib, and further still after the second (p<0.001). Notably, dexamethasone merely prevented their rise after day 9, which correlated with the mild reduction in plasma cell survival observed by day 14.

With irradiated cells from the same thymi, overall plasma cell survival and anti-AChR and total IgG productivity (see Fig. 6.3*A*-*C*) largely paralleled those from the untreated cells (Fig. 6.2*B*-*F*). Antibody production was generally lower, but was completely unaffected by dexamethasone, whereas it was again inhibited almost completely by bortezomib, as was total IgG production (Fig. 6.3*B, C*).

When all results were combined, numbers of plasma cells (whether irradiated or not) correlated strongly with spontaneous secretion of total IgG (ρ=0.89; p<0.0001) and AChR antibodies (ρ =0.79; p<0.0001).

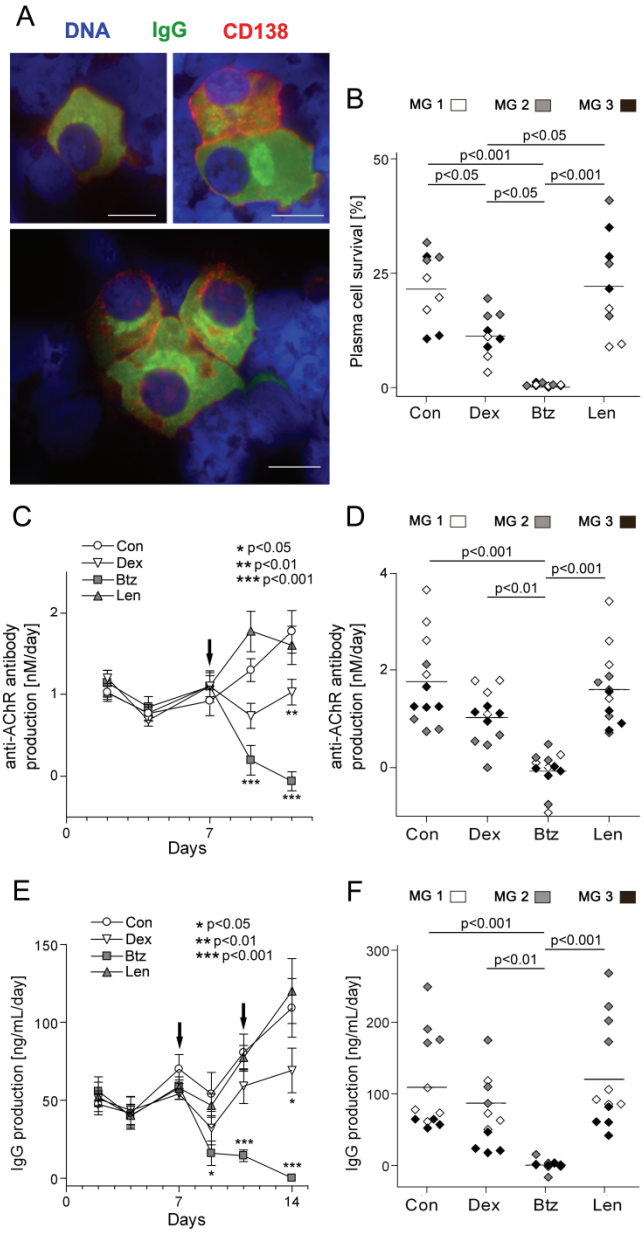

Figure 6.2. Changes in plasma cell numbers and functions after addition of bortezomib (2.5 µM), lenalidomide (10 µM) or dexamethasone (10 nM) on days 7 and 11, and collected on day 14. (*A*) Representative plasma cells from cytocentrifuged thymic cells. They were readily identified by their staining for IgG (green) and CD138 (red; DNA is blue), and typical morphological features, including: relatively large size, abundant cytoplasm with positive staining for immunoglobulins and eccentric nuclei. Frequently, plasma cells were found in clumps of 2-3 or more cells together, as shown in the lower panel. Scale bars are 10 µm. (*B*) Survival of plasma cells in cell cultures, expressed as a percentage of the input plasma cell numbers. Each point represents an individual well (3 per patient) and results correspond to day 14. (*C, D*) Spontaneous secretion of anti-AChR antibodies *in vitro*, measured with an immunoprecipitation radio-immunoassay and expressed as nmol of newly synthesized anti-AChR antibody/L/day. Results in *D* correspond to day 11. (*E, F*) Spontaneous secretion of total IgG *in vitro*. The IgG-content in the supernatant was quantified by a sandwich ELISA and results are expressed as ng of newly synthesized IgG/mL medium/day. Results in *F* are for day 14. Arrows indicate the days of drug addition. Each point represents the average of samples from all patients (4 per patient; *C, E*) or an individual well (*B, D, F*), and error bars (in *C, E*) correspond to the SEM; horizontal bars (*B, D, F*) represent the average of the group. One way (*B, D, F*) or two-way (*C, E*) ANOVA and Bonferroni post-hoc testing were used for statistical analysis.

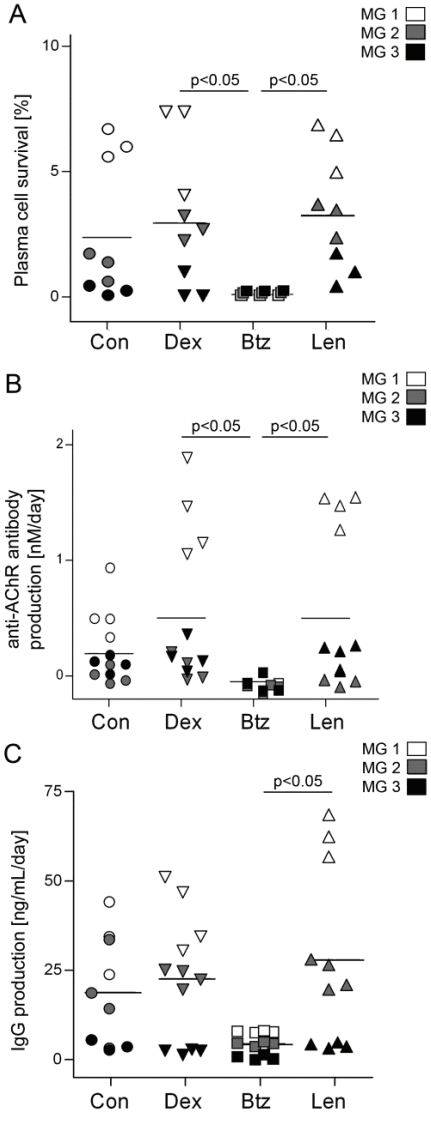

Figure 6.3. Susceptibility of irradiated cells to experimental drugs. Thymic cells were irradiated and subsequently cultured for two weeks. Drugs were added on days 7 and 11 (bortezomib - 2.5 μM, lenalidomide -10 μM, dexamethasone - 10 nM) and samples were collected and analyzed as for Fig. 6.2 at day 14. (*A*) Plasma cell survival. Although it was generally lower than with their unirradiated counterparts (Fig. 6.2*A*), the surviving plasma cells were still resistant to dexamethasone and lenalidomide, but clearly sensitive to bortezomib. (*B*) AChR autoantibody secretion. (*C*) IgG secretion. Each point represents an individual well. Grey shades indicate from which patient the sample originates. Horizontal bars represent the average of the group. One-way ANOVA and Bonferroni post-hoc testing were used for statistical analysis.

To test for effects on other cell types, we next stained unirradiated cultured cells for CD3 and CD19. Both dexamethasone (at 10 nM) and bortezomib (at 2.5 μM) reduced the numbers of T- and B-cells after the first dose (day 7; $p<0.001$) – and to a similar extent (Fig. 6.4*D, F*), although dexamethasone had much less effect than bortezomib on antibody levels. By contrast, lenalidomide (at 10 μM) had no significant effect on either cell numbers or anti-AChR antibody/ total IgG production (Fig. 6.4*D, F*). Together with the strong correlation between total IgG production and plasma cell survival, these results deeply implicate plasma cells, rather than B-cells, in the spontaneous antibody production that we observed *in vitro*.

Dose-dependence of bortezomib and dexamethasone effects on total IgG production and plasma cell survival

We next tested broader concentration ranges of dexamethasone and bortezomib. Total

IgG productivity and plasma cell survival were both minimal in the presence of 100 nM - 10 µM bortezomib (Fig. 6.5*A, B*). In sharp contrast, dexamethasone had no significant effects, even at 1 µM. Subsequently, we tested lower concentrations of bortezomib to estimate its minimum effective dose. As illustrated in Fig. 6.5*C-D*, it eliminated plasma cells and their IgG production even at 10 nM. As above, plasma cell survival correlated strongly with IgG production in these experiments.

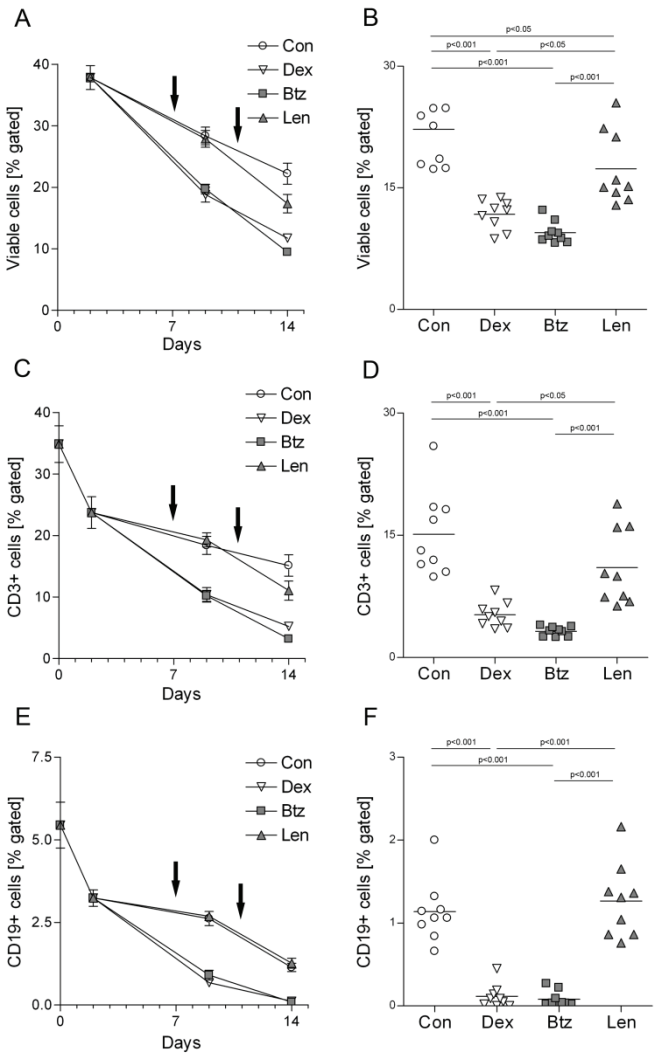

Figure 6.4. Quantitative overview of the drug effects on B- and T-lymphocytes. Thymic cells were cultured for 2 weeks and drugs were added on days 7 and 11 (indicated by arrows). Cells were collected and labeled for FACS analysis on days 0, 2, 9 and 14. Samples were gated to exclude cell debris, selected for PI-negative cells (viable cells) and for either CD3 or CD19. Results in panels B, D and F are for day 14. Each point represents the average of samples from all patients (3 per patient; A, C, E) or an individual well (B, D, F), and error bars (in A, C, E) correspond to the SEM; horizontal bars (B, D, F) represent the average of the group. One-way ANOVA and Bonferroni post hoc testing were used for statistical analysis.

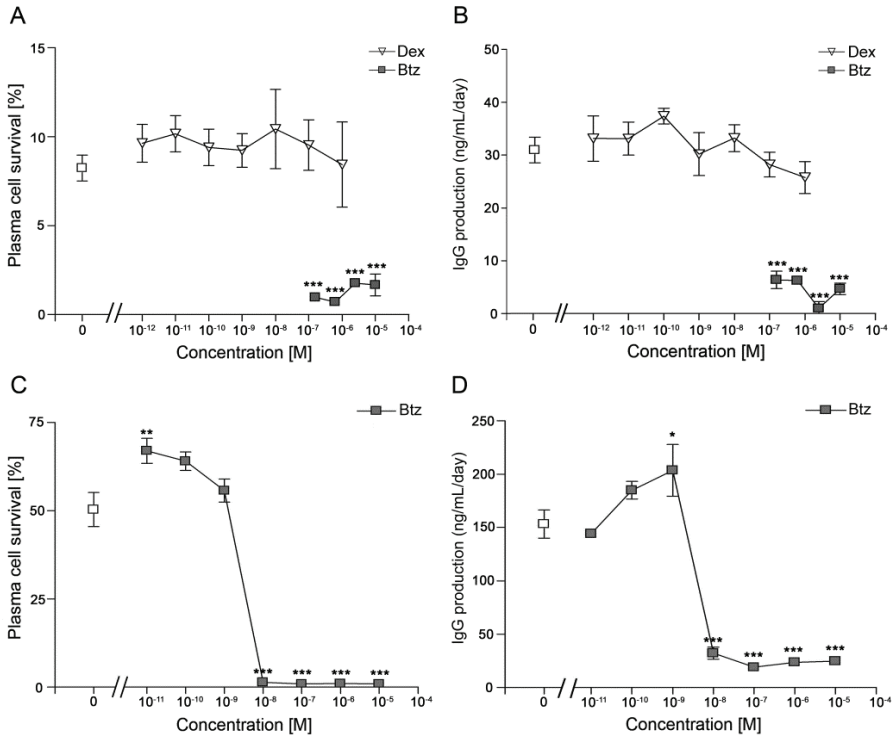

Figure 6.5. Dose-response curves for plasma cell survival (A, C) and total IgG secretion (B, D), after addition of drugs on day 4 and assay on day 7. Thymic cells from MG-1 were used in A and B; cells from MG-3 in C and D. Each point represents the average of 4 replicates and error bars the SEM. One-way ANOVA and Bonferroni post-hoc testing were used for statistical analysis. *p<0.05; ***p<0.01; ***p<0.001, compared with the control samples (white square).

To assess their general toxicity, we sampled cultures at earlier times after addition of these drugs. Cells collected at 6, 24 and 48 h showed no significant differences in overall viability. At day 7, viabilities were reduced more by dexamethasone at 1 µM than 10 nM; still more by bortezomib at 2.5 µM, but not significantly at 10 nM, where its effects were more selective for plasma cells (Fig. 6.1S). Surprisingly, lenalidomide inhibited IgG production only at 1 mM, when it also reduced total cell viability by an extra 40% (not shown).

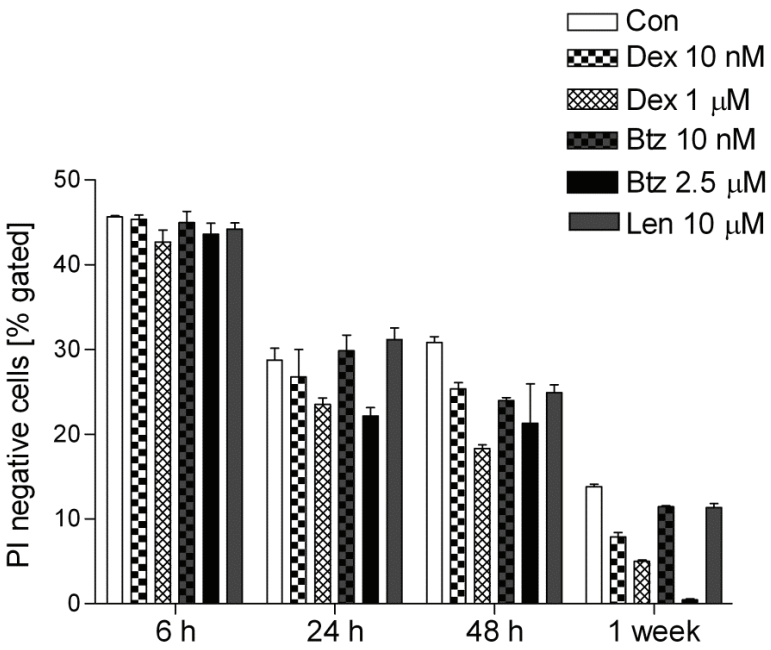

Figure 6.1S. General toxicity of the experimental drugs on thymic cells. Thymic cells from patient MG 4 were cultured for 1 week in the presence of different concentrations of the experimental drugs. Cell viability was evaluated at 6 h, 24 h, 48 h and 1 week by measuring propidium iodide (PI) incorporation by flow cytometry; samples were gated in the FSC-SSC dot-plot to exclude membrane debris. Cell viability decreased with time in all the conditions and was particularly low after 1 week exposure to dexamethasone (10 nM and 1 μM) and bortezomib 2.5 μM, but not bortezomib (10 nM) or lenalidomide (10 μM). Each bar represents the average of 3 replicates and error bars the SEM.

Discussion

In this study we demonstrate that bortezomib selectively eliminates long-lived autoimmune plasma cells in cultures of thymus cells from EOMG patients. Their spontaneous AChR autoantibody and total IgG production were completely halted, even at 10 nM. At 0.25 and 2.5 μM – and within 8 hours – it led to ultra-structural changes in plasma cells that are characteristic not only of ER stress but also of apoptosis, as seen *in vivo* too [23]. These are the first demonstrations of its efficacy directly on pathogenic autoantibody-producing human plasma cells. Since bortezomib clearly triggers apoptosis

in non-neoplastic plasma cells, even after a single dose, short low-dose regimens might be sufficient to rapidly reduce their numbers and antibody levels in patients with autoimmune disorders, thus 'buying time' during induction of immunosuppression with standard drugs.

In our cultures, plasma cells seemed almost entirely responsible for production of both autoantibody and total IgG, which correlated strongly with their survival. Moreover, it was maintained despite the depletion of B-cells by the addition of dexamethasone (Fig. 6.4*E, F*) – which evidently contributed minimally in untreated cultures (if at all). Indeed, we have rarely found signs of mitogen-stimulable production of anti-AChR antibodies in EOMG thymi [32]. These data, together with the antibodies' highly mutated heavy and light chain V region sequences, suggest that native AChR – which is continuously available in the thymus [31] – is driving most antibody-producing cells to the terminal plasma cell stage. We saw very similar behavior in remnant thymus tissue adjacent to thymomas [31], implying that our findings are not unique to EOMG.

In previous studies, plasma cells from recently immunized mice died after only 4 days in suspension cultures [48]. In sharp contrast, their Ig production was sustained *in vitro* for 2 - 4 weeks in primary cultures from human tonsils and gut-associated lymphoid tissue, and was enhanced by feeder cells and especially in whole organ cultures [49, 50]. It seems unlikely that equivalent survival niches were reconstituted efficiently in our cultures of cryostored, and especially irradiated, thymic cells. We suggest that the remarkable survival of the long-lived plasma cells shown here is due to their co-clustering (Fig. 6.2*A*) and/ or to some degree of resilience or 'self-sufficiency'. Their radio-resistance clearly shows that they are well established in many EOMG thymi and scarcely replaced in culture. Importantly, since bortezomib (but not dexamethasone) reduced their numbers and antibody production to baseline in irradiated samples, its targets must include long-lived plasma cells.

In sharp contrast with our results with bortezomib, but in agreement with previous *in vivo* findings [24, 51], we found only marginal effects of dexamethasone on plasma cell survival or function, even when added twice at 1 μM, and only on unirradiated cells. Evidently, most of the thymic plasma cells are dexamethasone- as well as radio-

resistant; indeed, neither treatment completely eliminated them in any of our cultures. In theory, both treatments might also affect their supporting cells, and/ or damage other short-lived plasma cells or plasmablasts. *In vivo*, however, their precursors may be steroid-insensitive too; we noted no obvious decrease in PWM-stimulated IgG responses by (radio-sensitive) B-cells from prednisone-pretreated patients – rather, they appeared to be enriched [52].

The even smaller effects on plasma cells of the immunomodulatory drug lenalidomide may seem surprising in view of its clear benefits in MM patients [53]. One possible explanation is that its toxicity for MM cells is mainly related to the activation of tumor suppressor genes and caspases that trigger apoptosis in transformed cells [54-56], but probably not in their non-neoplastic counterparts. Moreover, since lenalidomide disrupts the survival niches required by MM and plasma cells, such toxic effects may be under-estimated in our cultures. In addition, it is well-known for its disparate immunomodulatory properties, e.g., inhibiting Ig production by cultured PBMCs [30], but also augmenting antibody responses to vaccination [57], and inducing proliferation and activation of T-cells [29, 58]. Taken together, our *in vitro* results indicate that, unlike MM cells, non-neoplastic plasma cells are not directly killed by lenalidomide. However, its possible effects on their niches *in vivo* might valuably complement the direct actions of bortezomib in patients [42].

Both bortezomib (at higher concentrations) and dexamethasone reduced CD19 and CD3 lymphocytes in our cultures. This is in line with the reported effects of bortezomib on activated human B-cells [59] and total circulating B-cells in EAMG rats [23]. Moreover, bortezomib influences T-cell subset distributions, inducing apoptosis in activated CD4 T-cells, preventing the activation of memory T-cells [60], but preserving resting and regulatory T-cells [61-63], and promoting their *de novo* generation [63]. Additional effects of bortezomib on activated B- and T-cells, or on antigen-presenting B-cells, could be an advantage in treating MG patients, e.g., in preventing the generation of new auto-reactive plasma cells while also eliminating the existing long-lived subset.

Although bortezomib is clearly effective in treating MM, SLE, TTP and acute antibody-mediated transplant rejection, there are serious concerns about such side-

effects as thrombocytopenia and, especially, peripheral neuropathy. These may well correlate with the long courses and high doses (1.3 mg/m^2; resulting in plasma levels of C_{max} = 600 nM [40] of bortezomib and other chemotherapeutics needed to eliminate as many MM cells as possible in patients. MM and related leukemia cell lines are susceptible to bortezomib with an IC_{50} of 12 nM *in vitro* [64] which is comparable to the 10 nM that proved effective here. Since even partial elimination of autoimmune plasma cells might be sufficient to treat MG, the side-effects might be avoided in autoimmune patients by using shorter courses and/ or lower doses of bortezomib, e.g., in combination with plasma exchange [65, 66]. Our results suggest that these should rapidly deplete plasma cells/ reduce antibody levels in the short-term – as now seen in prevention of hyper-acute antibody-mediated transplant rejection [21, 67]. Indeed, adverse effects were significantly fewer with such 'light-touch' regimens [40, 65, 66]. Finally, some second generation proteasome inhibitors have equal or greater potency but lower neurotoxicity than bortezomib, and are already being tested in clinical trials [68].

In conclusion, our study using EOMG thymic cells, in combination with our previous results in the EAMG model [23], gives proof-of-principle for using proteasome inhibitors for the elimination of non-neoplastic plasma cells in autoantibody-mediated disorders. This therapeutic strategy could have potential applications in AChR-MG, especially for rapidly reducing autoantibody titers and so temporizing while standard immunosuppressants start to take effect, and/ or in treatment-refractory patients. However, these potential benefits in MG or other antibody-mediated diseases still need to be balanced very carefully against their side-effects.

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General Discussion

Alejandro M. Gomez

Plasma cells and autoantibody titers in myasthenia gravis

Although it is widely accepted that MG is mediated by autoantibodies directed against postsynaptic proteins of the NMJ (the AChR, in most cases), the correlation between autoantibody titers and severity of symptoms has remained controversial to demonstrate in large cohorts of patients [1-4]. Nonetheless, it is generally acknowledged that the fluctuations in anti-AChR titers do correlate with the severity of symptoms for a given individual patient, implying that they could represent a valuable tool for evaluating responses to treatment, in addition to the MGFA score [5]. One possible explanation for these discrepancies relies on the differential specificities or affinities of anti-AChR antibodies among MG patients. In this regard, it has been shown that most MG patients produce autoantibodies directed against the main immunogenic region of the AChR (anti-MIR antibodies), though such anti-MIR antibody titers do not correlate well with disease severity [4]. However, it has been recently reported in a large cohort of MG patients that anti-MIR antibody titers do have a positive correlation with clinical factors typical of generalized and severe MG, and a negative correlation with those of ocular MG, rendering them useful for differential diagnosis between ocular and generalized MG [6]. Thus, even though several elements are involved in the severity of symptoms in MG (e.g. complement, response of the muscle to the autoimmune attack, cytokine levels), autoantibody titers seem to be one of the most directly related to disease severity and, consequently, their reduction remains an essential therapeutic goal in MG. Accordingly, it has been demonstrated by several studies that reductions of about 50-75% in autoantibody titers correlate well with clinical improvement for a given patient [1, 7, 8].

There are several immunosuppressive therapies currently available that will eventually reduce autoantibody titers in most of MG patients. However, they usually require a prolonged induction period (e.g. azathioprine and, to a lesser extent, corticosteroids), which increases morbidity and the chances for undesired side-effects. A rapid reduction of autoantibody titers becomes particularly important during a myasthenic crisis and, until now, there are no better alternatives to achieve this rapid reduction than plasma exchange or IVIg; which offer only temporary relief. Moreover,

some MG patients are refractory to most of the standard treatments available (even to new ones such as the anti-CD20 monoclonal antibody rituximab), and are thus in need of novel treatment alternatives. As discussed in Chapter 4, targeting plasma cells (particularly the long-lived plasma cells, which can resist most of the immunosuppressive drugs [9-11] and even irradiation [10]), could offer a novel and valuable approach for both rapidly reducing autoantibody titers and for treating refractory MG patients.

Following this idea, I demonstrated in Chapter 5 that the proteasome inhibitor bortezomib significantly depletes bone marrow plasma cells in the EAMG model [12], leading to a rapid and significant reduction of autoantibody titers. Since the bone marrow is considered to be the main site for homing of long-lived plasma cells [13-15], depletion of bone marrow plasma cells by bortezomib is likely to reflect effects on the long-lived subpopulation. In that study, EAMG-rats were treated with bortezomib twice a week for 8 weeks in two treatment schemes: one starting directly after immunization with *Torpedo* acetylcholine receptor (tAChR) (8w-Bz); and the other 4 weeks after immunization (4w-Bz), once autoantibodies against the AChR were already present in serum. After 8 weeks of treatment, a significant reduction in both plasma cells numbers and autoantibody titers was observed in bortezomib-treated rats in comparison with saline-treated animals and, importantly, no differences were observed between the 8w-Bz and 4w-Bz treatment schemes. Therefore, proteasome inhibition could both prevent the development of EAMG and also significantly reduce autoantibody titers once the immune response has already started. This rapid reduction of antibody titers correlated well with the significant depletion of plasma cells observed after treatment with bortezomib in both 4w- and 8w-Bz groups, indicating that indeed killing the plasma cells can rapidly reduce antibody titers (i.e. in less than 4 weeks). Lower autoantibody titers led to improved clinical status for most of 8w-Bz animals. The clinical scoring of 4w-Bz animals, however, was not significantly improved despite their significant autoantibody reduction, which was comparable to that of 8w-Bz animals. This is most likely explained by transient acute side effects of bortezomib administration (weight loss and possible direct effects on neuromuscular transmission) that occurred earlier in 8w-Bz animals and, consequently, affected less their clinical symptoms at the endpoint than in 4w-Bz animals. Also, the NMJ

in 4w-Btz animals was probably severely affected by the time of bortezomib administration and, considering that it requires several weeks for the NMJ to fully recover after an autoimmune attack [16], the mild clinical recovery after 4 weeks of treatment was possibly due to a yet incomplete repair of NMJs. Had I treated these animals for a longer period, I might have found greater improvements in their clinical condition.

The toxic effects of proteasome inhibition on plasma cells were confirmed and further characterized in thymic cell cultures from EAMG patients that underwent thymectomy (Chapter 6). I demonstrated that bortezomib, but not corticosteroids or lenalidomide, can virtually eliminate human autoimmune plasma cells cultured *in vitro* in less than 24 h. This results in a complete blockade of autoantibody and total IgG production in these cultures, even at lower concentrations than those normally used in *in vitro* studies or the peak values found in patient's sera. Moreover, ultrastructural alterations suggestive of apoptosis were found in plasma cells by electron microscopy as early as 8 h after administration of bortezomib, corroborating qualitative observations in our *in vivo* study (Chapter 5). Even though these findings are *in vitro* and, most likely, the *in vivo* kinetics and efficacy of bortezomib-induced plasma cell death might be different, as already suggested in Chapter 5, they certainly support the notion that killing plasma cells leads to a rapid and effective reduction in autoantibody production.

Moreover, bortezomib had toxic effects on lymphocytes in both the EAMG model and in cultured EOMG thymic cells (Chapters 5 and 6, respectively). I found a reduction in circulating B-cells in bortezomib-treated rats (both 4w-Bz and 8w-Bz) and a significant depletion of both T- and B-cells in thymic cell cultures 48 h after administration of bortezomib. In this regard, previous studies had shown that bortezomib affects the dynamics of different lymphocyte populations: it induces apoptosis in activated CD4+ T-cells, but preserves inactive and regulatory T-cells [17-19]; it promotes the generation of new T-suppressor cells [19]; it also prevents the activation of memory T-cells [20]; and it induces apoptosis in activated B-cells [21]. At present, it is quite accepted that mature, and even memory, B-cells are not crucial for the maintenance of antibody levels once long-lived plasma cell have been generated [15, 22]. However, additional effects of bortezomib on activated (and memory) B- and T-cells could be an advantage in treating

MG patients, since this can help to prevent the generation of new auto-reactive plasma cells while also eliminating the existing long-lived subset.

Generally speaking, our results neatly complement observations from trials of rituximab in MG patient sub-groups [23] and in SLE [24], where autoantibody titers remained constant for at least one year after complete depletion of circulating B-cells, probably because of ongoing production by long-lived plasma cells. In this regard, the reduction in bone marrow plasma cells in bortezomib-treated animals (~60%) correlated well with the reduction in autoantibody titers (~72%) and total IgG (~50%) in the animals' serum (Chapter 5), which further supports the hypothesis of the (largely) long-lived bone marrow plasma cells as major contributors to (auto-) antibody levels in AChR-MG. By contrast, contributions from B-cells and short-lived plasma cells are apparently greater in the minority of MG patients with anti-MuSK antibodies, where rituximab treatment led to both long-lasting clinical improvements and sharp reductions in antibody titers [23]. Evidently, the underlying responses differ greatly in these two distinct autoimmune disorders, although both finally lead to myasthenia. Indeed, the thymus is scarcely involved in MuSK-MG [25], where pathogenic autoantibodies are mainly IgG4, in contrast with the IgG1 and IgG3 in EOMG. The predominantly-IgG4 autoantibody response in MuSK-MG is apparently sustained mostly by short-lived plasma cells that are continuously regenerated from autoreactive $CD20^{+}$ B-cells [23], whereas long-lived plasma cells may play a more dominant role in AChR-MG.

Models to explain humoral immunological memory

Humoral immunological memory is fundamental for the survival of higher vertebrates and it has probably evolved as a tool to avoid re-infection with previously encountered pathogens and to cope with the host's microbiota [26, 27]. The concept of immunological memory has been implicitly known for centuries: already in the 10^{th} century Chinese practitioners inoculated dried scabs from smallpox pustules in the nostrils of naïve recipients to prevent infection (variolation). However, its mechanisms and regulation have remained largely unknown. Only recently the scientific community started to genuinely study the biology of immunological memory, the types of cells involved, the dynamics of

vaccination protocols, etc. For instance, such a basic concept as the duration of antibody responses upon infection or vaccination was not seriously addressed until the 2000's. In a longitudinal analysis of antibody titers to vaccination antigens in the general population, Amanna et al. estimated the half-life of antibody responses to range from 11 years (for tetanus toxoid) up to centuries (for measles and mumps) [28]; implying that the humoral immune system is able to provide protection against re-infections, at least for certain antigens, for a lifetime.

Traditionally, humoral immunological memory was thought to rely on the generation and re-activation of memory B-cells upon re-encounter with an antigen. These models, however, started to be challenged in the 1990's since they could not fully explain the duration and kinetics of antibody responses. Memory B-cell-based models explain prolonged antibody production in terms of persistent antigens (chronic viral infections), repeated exposure to antigen (re-infection or booster vaccination), cross-reactivity and polyclonal activations [29], among others. However, for most of these assumptions, there are solid arguments that point against memory B-cell-only sustained antibody responses. First, a clear correlation between frequency of memory B-cells and antibody titers have been difficult to demonstrate for several antigens [28, 30]. Second, reactivation of latent chronic viruses such as the varicella zoster (shingles) increases antibody titers for the virus, but they will subsequently decrease back to basal pre-reactivation levels in the following years, which is also the case for antibody titers after booster vaccination [28, 31]. This suggests that there is a physiological threshold for the production of antibodies to a certain antigen that helps to keep "basal" antibody levels relatively constant in time [29]. Third, complete elimination of circulating B-cells with an anti-CD20 mAb scarcely affects total IgG levels or antibody levels against vaccination or previously encountered antigens [11, 32]. Fourth, the half-life of antigens in antigen-presenting cells is about 2 months [33] and, consequently, antigen-driven constant B-cell re-activation cannot explain sustained antibody production for several years. And finally, booster vaccination has little to no effect on antibody titers to other antigens, indicating that random polyclonal activations of memory B-cells are not likely to replenish the pool of plasma cells and, thereby, maintain antibody titers for longer periods of time [28, 29]. It seemed thus, that a piece of

the puzzle was missing in these models in order to comprehensively understand humoral immunological memory: the long-lived plasma cells.

Long-lived plasma cells are nowadays considered as a fundamental and largely memory B-cell independent compartment of immunological memory, with its own homeostasis and regulatory mechanisms [11, 22, 34, 35]. One of the most widely accepted models of long-lived plasma cell survival proposes that, in order to be long-lived, plasma cells must occupy special survival niches in immune organs such as the bone marrow or the spleen [36, 37]. Since the number of such niches is limited, newly-generated plasma cells have to displace older plasma cells from their niches in order to survive. Thus, such model predicts that, for every new immune response against a certain antigen that produces a wave of plasma cells, there will be decays in the antibody levels to previous antigens as a consequence of displaced long-lived plasma cells. This, however, does not seem to be the case based on a study that followed antibody responses for decades and showed very constant antibody levels against various antigens; even at old ages, when the number of survival niches available is believed to be significantly reduced [28]. More recently, a new complimentary model was proposed in which plasma cells would be imprinted with the capacity for long-term survival ("competence to compete"), though they still must reach a survival niche in order to survive ("competition for survival") [38, 39]. Such imprinted capacity is believed to be acquired at the moment of B-cell activation, prompted by interactions with T-helper cells at the germinal center. Under this new concept, immunological memory to a certain antigen due to long-lived plasma cells would be the result of both how efficient is the immune response to elicit a substantial number of long-lived imprinted plasma cells, and how successful are they in homing to survival niches; either by displacing previously-generated plasma cells or by occupying (or perhaps even inducing) new survival niches. The advantage of this model is that only immune responses that occur with substantial T-helper cell intervention in the finely-regulated environment of the germinal center can provide long-lived plasma cells [29]. This process would allow for a "fine-tuning" in the selection of valuable antibody responses, ensuring that not every plasma cell that is generated can occupy survival niches and, thus, maintain an antibody response against antigens that are not likely to be relevant in the future.

Long-lived plasma cells in myasthenia gravis

Given their fundamental role in the maintenance of long-term antibody responses, and their well-known resistance to most of the immunomodulatory treatments currently available, long-lived plasma cells have become a promising therapeutic target in autoimmune diseases [40-42]. It is certainly possible that autoimmune responses generate long-lived plasma cells that, once in their survival niches at the bone marrow, can perpetuate the production of autoantibodies. This was clearly demonstrated, for instance, in a mice model of systemic lupus erythematosus in which autoantibody production was shown to be driven by both short- and long-lived anti-DNA splenic plasma cells [43]. Also in inflammatory autoimmune diseases, inflamed tissues can provide new survival niches that can accommodate (long-lived) auto-reactive plasma cells generated *in situ* [44], as it was suggested for the synovial tissue of rheumatoid arthritis patients [45] and demonstrated for the kidney of NZW/B lupus mice [46].

Since plasma cells – especially the long-lived subset – are thought to be highly dependent on special niches in the bone marrow and lymphoid tissues, it is remarkable that they survived and secreted so well even in our suspension cultures of cryostored cells (Chapter 6). Their frequent appearance in clusters (Chapter 6, Fig. 2A) may imply some reconstitution of these niches, but it was unlikely to have been optimal, especially with irradiated cells. Previous studies have shown that primary cultures of plasma cells from human lymphoid organs, such as tonsils and the small intestine gut-associated lymphoid tissue (GALT), can sustain antibody production *in vitro* for up to 2 or 4 weeks, respectively [47, 48]; a feature that was enhanced by the presence of feeder cells and, even more, by maintaining the intact tissue in culture. In our samples, plasma cells could still survive and spontaneously produce antibodies for at least two weeks (and up to 4 weeks, data not shown) despite disruption of the normal tissue architecture, which implies that the supportive cells required for plasma cell survival were still active after enzymatic disruption, and even after irradiation.

Several cellular and molecular components of the survival niches have been proven to contribute to plasma cell survival (e.g. stromal cells, eosinophils, monocytes,

macrophages, IL-5, IL-6, TNF-α, SDF-1α, hyaluronic acid, APRIL, CXCL12, BAFF) [34, 37, 49, 50], while most of them primarily act by synergistic effects and no single mediator seems to be *per se* essential for plasma cell survival (their function is many times redundant). Furthermore, plasma cells have been shown to induce other cell types in their vicinity to secrete pro-survival cytokines (e.g. stromal cells to produce IL-6 [51]), in what could be a feedback system for the generation and maintenance of survival niches [52] . Thus, the sustained presence of long-lived plasma cells in our cultures might be explained by this apparently flexible nature of the survival niches, since the potential loss of redundant pro-survival signaling (i.e. loss of supportive cell types or cytokines due to tissue disruption or irradiation) can be probably compensated by other cell types remaining in EOMG thymus cultures.

As mentioned, the generation of long-lived imprinted plasma cells is highly dependent on interactions between the antigen, the B-cell receptor and T-cells in the context of the germinal center [52]. Depending on the nature of the antigen and, therefore, the degree of T-helper co-stimulation, short- or long-lived antibody responses will be generated. On the one hand, if the antigen has no repetitive epitopes to cross-link the B-cell receptor and induces none or poor T-cell activation, then the immune response is likely to remain short-lived (<3 years). On the other hand, if the antigen has repetitive epitopes that can cross-react the B-cell receptor, and also activates T- helper cells, then the immune response has the potential of producing a substantial number of long-lived plasma cells [29]. In antibody-mediated autoimmune diseases such as MG this might well be the case, at least for some patients. For instance, the sustained autoantibody titers in refractory AChR-MG patients depleted from B-cell with long-term Rtx treatment is likely to be produced mainly by long-lived plasma cells [23], as previously discussed. Also, the enrichment of anti-AChR T-cells and B-cells in the PBMCs of MG patients [53, 54] clearly indicates that auto-reactive germinal center can be formed, leading to affinity maturation of autoantibodies and generation of potentially long-lived plasma cells.

Accordingly, one of the hallmarks of MG-thymus is the presence of active germinal centers [55, 56], which are normally absent in control thymi, and that contain all the ingredients to generate long-lived autoimmune plasma cells: anti-AChR T- and B-cells,

and the locally-expressed AChR in myoid and thymic epithelial cells [57, 58]. Indeed, anti-AChR germinal centers and plasma cells are frequently found in the thymus of EOMG patients and, once in culture, the latter can substantially secrete pathological autoantibodies for several weeks [59] (Chapter 6), suggesting that they are long-lived. Consequently, the loss of these thymic autoimmune long-lived plasma cells and/or thymic auto-reactive germinal centers after thymectomy could be an explanation for the reduction of anti-AChR titers, improvement of clinical condition, or even remission of some thymectomized MG-patients [60-65].

The muscle's response to an autoimmune attack: implications for disease severity

As explained in Chapter 1, the pathophysiology of AChR-MG is well characterized and the pathogenic role of anti-AChRs has been clearly demonstrated. Still, an important feature of MG patients is their substantial heterogeneity of symptoms and variability in clinical severity [66]. Although these characteristics have been widely acknowledged in several studies and are well categorized [67, 68], up to date, their underlying causes remain poorly understood. As previously discussed, differences in anti-AChR antibodies specificity, affinity, or titers could offer an explanation to the variability in severity among MG patients but these correlations have, for the most part, remained controversial and, therefore, inconclusive [1-3, 5, 6]. Alternatively, differences in the response of the muscle, in particular of the NMJ, to the autoimmune attack might account for the variability in disease severity in MG. Such intrinsic differences in the muscle's response, in combination with the variability of the autoimmune response, could provide a more comprehensive explanation to the differential development of symptoms in MG; for instance, they most certainly account for the involvement of certain muscles (e.g. ocular muscles) in preference to others [69]. Moreover, it was reported that about half of MG patients in clinical remission (absence of symptoms without therapy) have still electrophysiological abnormalities and sustained presence of anti-AChR antibodies [70]; suggesting that, so far, unknown factors in these patients (e.g. intrinsic properties of the muscle, variability in the complement system, or in regulators of complement activation such as DAF) might

account for this lack of symptoms despite the presence of pathological abnormalities. In addition, understanding the response of the muscle in MG could offer new insights into potential therapeutic options to increase the tolerance of the NMJ to an autoimmune attack.

To study the proteins involved in the response of the muscle to an autoimmune attack, I analyzed the proteome of rat tibialis anterior muscles that have been chronically challenged by anti-AChR antibodies in the EAMG model (Chapter 2). In this study, I observed that most of the differentially-expressed muscle proteins were cytoplasmic and related to metabolic pathways, cellular-stress responses or contractile proteins. Generally speaking, there was a reduction in the glycolytic capacity of the muscle and a shift towards expression of slow-twitch fiber isoforms of contractile proteins. Together, these findings indicated that the muscle in EAMG animals is either preferentially losing fast-twitch fibers or is actively switching to slow-type fibers, or a combination of both. In this regard, it has been described that muscle biopsies from MG patients have also preferential atrophy of fast-twitch fibers [71-73].

Since fast-twitch fibers rely primarily on anaerobic ATP generation through glycolysis, in contrast to aerobic mitochondrial ATP generation in slow-twitch fibers, they are more prone to fatigue than their slow-type counterparts [74]. Additionally, intrinsic contractile properties of fast-twitch fibers (e.g. differential expression of heavy chain myosin isoforms) also renders them susceptible to fatigue [75]. Taking into consideration that functional denervation of muscle fibers typically results in preferential atrophy of fast-twitch fibers [76] one possibility is that, in both MG and EAMG, the impairment in neuromuscular transmission is less tolerated by fast-twitch fibers and, consequently, these fibers are preferentially lost. This phenomenon could be attributed to an increased sensitivity to inactivity or higher neurotrophic dependence of fast-twitch fibers [77, 78], though this has not been clearly demonstrated yet [79]. Alternatively, since muscles with a majority of slow-twitch fibers are more resistant to fatigue [80], the preferential loss of fast-twitch fibers might be a response of the tissue to counteract the muscle fatigability that results from poor neuromuscular transmission. In MuSK-MG patients, for instance, it has been reported that fast-twitch muscles are preferentially affected [81], suggesting

that they are more prone to experience weakness and fatigability in the event of poor neuromuscular transmission than slow-twitch muscles. Also, the peculiar fiber composition, mechanical properties, and myosin heavy chain expression of extraocular muscles are, arguably, the main reasons behind their preferential affection in MG [69]; which further implies intrinsic muscle properties as fundamental factors for the development of symptoms.

Unexpectedly, I could not detect any NMJ-protein differentially expressed in EAMG muscles by a 2D-DIGE proteomics approach (Chapter 2). On the one hand, given the well-known loss of AChR and AChR-related proteins in EAMG, this was rather counterintuitive. On the other hand, technical limitations of 2D-DIGE proteomics for detecting membrane-bound proteins (i.e. poor solubility of these proteins in buffers used for isoelectric focusing) [82], and the fact that NMJ proteins represent a very small fraction of the muscle's proteome, can possibly explain their absence in our analysis. In this regard, NMJ proteins were also not satisfactorily detected in more comprehensive studies that characterized the whole muscle's proteome with more sensitive technological approaches [83]. Possibly, in the near future, novel techniques such as shotgun proteomics, subcellular fractionation, free-flow electrophoresis, and others, might overcome the limitations for detecting membrane-bound proteins in proteomics studies [84] and, consequently, provide better tools for investigating the response of the NMJ to an autoimmune attack.

Upon binding of anti-AChR antibodies at the NMJ, complement is locally activated and loss of AChRs and AChR-associated proteins occurs (reviewed in Chapter 1). In EAMG models, a reduction in extracellular AChR-associated proteins such as utrophin [85] and MuSK (unpublished observations), in addition to intracellular proteins such as rapsyn [86], have been reported. These proteins are all implicated in the clustering and maintenance of AChRs at the adult NMJ, and their loss could contribute to delay the repair processes of the endplate after an autoimmune attack (reviewed in Chapter 1). In this regard, I studied the effects of down-regulating the expression of Dok-7, the most recently described member of the AChR clustering pathway, in the recovery from and susceptibility to the EAMG model (Chapter 3).

Dok-7 is an intracellular adaptor protein that is required for full activation of

MuSK, the central kinase in the organization of the muscle's postsynaptic apparatus, and its presence is fundamental for the fetal development of neuromuscular synapses. Contrary to my expectations, silencing of Dok-7 expression did not significantly delay the recovery from the damage caused by 20 pmol mAb35/100 g body weight (anti-AChR antibody), probably because I underestimated the time needed for a full functional recovery of the NMJ (Chapter 3). However, Dok-7 silencing did increase the susceptibility to EAMG, when it was induced with a subclinical dose of mAb35 (5 pmol/100g body weight), suggesting that an impaired clustering machinery renders the NMJ more prone to AChR loss in the case of an autoantibody attack. These findings are in accordance with the clinical characteristics of patients with CMS due to Dok-7 mutations, since they have generalized muscle weakness and smaller, more disorganized, endplate regions [87-89]; a feature that I also observed in C2C12 myotubes silenced for Dok-7 expression (Chapter 3). In addition to a lack of MuSK activation, a reduction in Dok-7 levels might also influence downstream signaling cascades independently from MuSK signaling, since it was recently demonstrated that Dok-7 can bind Crk and Crk-L in its C-terminal upon phosphorylation [90]. These adaptor proteins are able to link the postsynaptic apparatus with the actin remodeling system and AChR clustering, thereby acting as integrating factors for multiple pathways [90].

A deeper understanding of the functioning of the AChR clustering machinery and the muscle's response to the autoantibody attack could prove useful for symptomatic treatment of MG patients. Overexpression of the anchoring protein rapsyn, for instance, has been shown to increase the resistance of the NMJ to the damage inflicted by autoantibodies in the passive-transfer EAMG model [91] and might even reverse the loss of AChRs in ongoing chronic EAMG [85]. Also, administration of a fast-skeletal-troponin activator (CK-2017357) was shown to increase muscle force in response to sub-optimal nerve signaling and, thereby, improve muscle strength in the passive-transfer EAMG model [92]. These studies are clear examples on how manipulations of muscle proteins can increase the tissue's tolerance to the autoimmune attack in MG and, consequently, they provide novel options for symptomatic treatment other than the traditionally used acetylcholinesterase inhibitors. Additionally, regulating the expression of NMJ proteins

with gene therapy, or modulating their activity with synthetic agents, might eventually be used to treat not only MG, but also CMS. Although rather experimental treatments such as gene therapy are still far from being a common practice in the clinic, much progress has been made during the past decades and several gene-therapy clinical trials have proven successful to treat human diseases [93-95]. Keeping these therapeutic options in mind, a more detailed knowledge about the repair and maintenance of adult NMJs is likely to be fundamental for the development of more targeted treatments for MG and CMS in the future.

Conclusions

The development of symptoms in MG results from the fine balance between the autoimmune response against the NMJ and the capacity of this synapse to resist an autoimmune attack, or repair itself after it. Traditionally, the treatment of MG has relied heavily on immunomodulatory agents that block the autoimmune response rather unspecifically and on acetylcholinesterase inhibitors (the only symptomatic treatment available). In this thesis, I have investigated new therapeutic strategies for targeting two of the pathophysiological components of MG that are most directly involved in the development of symptoms, and that have not been frequently taken into consideration so far: the plasma cells and the muscle.

Plasma cells are relevant in MG primarily because they are the main antibody-producing cell of the immune system and they resist most of the currently available treatments. I have shown in both the EAMG model and primary thymic cell cultures from MG patients that depletion of plasma cells rapidly and efficiently reduces autoantibody production, providing a strong proof of principle for its future use in patients. A characterization of the muscle's response to the autoimmune attack was also performed, in an attempt to analyze individual proteins involved in resistance or repair mechanisms at the NMJ. Despite the lack of NMJ-proteins differentially-expressed in the proteomics study of EAMG muscles, I found protein changes suggestive of a switch in muscle fiber composition, in what could represent compensatory mechanisms of the muscle to prevent weakness and fatigability. In addition, I have shown that reduced levels of the adaptor

protein Dok-7, involved in AChR-clustering, increases the susceptibility to passive transfer EAMG and, consequently, I provide a demonstration that levels of AChR-associated proteins can also influence disease severity in EAMG.

Expanding the treatment options for MG by focusing on traditionally neglected therapeutic targets might eventually prove beneficial for MG patients in the near future, particularly those who are refractory to the currently available therapies. Additionally, given that these treatment strategies are relatively restricted to target only components directly involved in the development of symptoms (autoantibodies and the NMJ), they can also be valuable to prevent undesired side-effects of the more unspecific therapies commonly used. As a consequence, the therapeutic strategies discussed in this thesis are, in a way, a step forward towards developing more specific therapies for MG and, on the whole, they have the potential of becoming valuable tools for the management of MG patients in the future.

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Summary

This thesis is aimed at investigating new strategies for a more comprehensive treatment of MG, a well characterized autoimmune disease of the NMJ that leads to muscle weakness and fatigability, which is typically treated by a combination of wide-range immunomodulatory agents. To address this issue, I focused on two factors that are not usually taken into account as therapeutic targets and that are directly involved in the development of symptoms: the plasma cells (the main producers of autoantibodies) and the response of the muscle to an autoimmune attack.

Chapter 1 explains the structure and functioning of the NMJ, emphasizing its electrophysiological and structural characteristics, and the corresponding pathological changes associated with MG. Particular interest is paid to the pathological mechanisms of the different subclasses of autoantibodies in the main subsets of MG: AChR-MG (predominantly pro-inflammatory subtypes IgG1 and IgG3) and MuSK-MG (predominantly anti-inflammatory IgG4). The ability to bind and activate complement, undergo Fab exchange, increase the antigenic modulation of AChRs, or functionally block the AChR is thoroughly explained for these IgG subtypes. In addition, the loss of several AChR-associated proteins which are important for neuromuscular synaptogenesis (utrophin, rapsyn, MuSK, Dok-7, Lrp4, Tid1) is discussed for both MG and CMS, and the concept of loss of AChR-associated proteins as a possible contributor to disease severity in MG is introduced. Finally, the main animal models of MG and the relative contribution of different IgG subtypes to these models are described.

Chapter 2 studies the response of the muscle to an autoimmune attack mediated by anti-AChR antibodies in the chronic EAMG rat model. A two-dimensional difference in-gel electrophoresis (2D-DIGE) was utilized to analyze the muscle's proteomic profile at different stages of EAMG. A total of 22 differentially expressed proteins, mainly related to metabolic and stress-response pathways, were identified. NMJ proteins were not found to be differentially expressed, most likely due to the relative low abundance of these proteins in the whole muscle's proteome and also because of technical limitations of 2D-DIGE for detecting membrane-bound proteins. Interestingly, the changes in differentially expressed proteins were suggestive of a switch of muscle fiber type towards more fatigue-resistant slow-twitch fibers, or a preferential loss of fatigue-prone fast-twitch fibers. This

phenotype has also been associated with other contraction-impairing muscle pathologies (e.g. inclusion body myositis), which suggests a similar response of the muscle to such conditions.

Chapter 3 investigates the role of the adaptor NMJ protein Dok-7, crucial for the embryonic development of NMJs and the full activation of MuSK, in a passive-transfer EAMG model. Since Dok-7 is the most recently described member of the AChR clustering machinery, and its mutations are one of the leading causes of CMS, it was hypothesized that reduced levels of Dok-7 at the NMJ increases the susceptibility to develop EAMG and impairs recovery after the antibody attack. To test this hypothesis, shRNAs targeting Dok-7 were transfected into the tibialis anterior muscle of adult rats by *in vivo* electroporation and EAMG was induced 35 days later. Silencing of Dok-7 significantly impaired neuromuscular transmission and reduced AChR levels at the NMJ in EAMG animals that received a subclinical dose of mAb35 (5 pmol/100g body weight). By contrast, silencing Dok-7 had no effects either on control animals or in the recovery from the autoantibody attack up to 14 days after induction of EAMG. At this time-point, however, the NMJ had still not fully recovered its functionality, unexpectedly. Additionally, no evident morphological abnormalities were observed by electron microscopy in Dok-7-silenced NMJs. These results suggest that a reduced Dok-7 expression is not essential for the recovery of the NMJ after damage but may play a role in the susceptibility to EAMG, probably by rendering AChR clusters less resistant to the autoantibody attack.

Chapter 4 discusses the potential therapeutic applications of plasma cell depletion with the proteasome inhibitor bortezomib in MG. This chapter elaborates on the relative contribution of plasma cells, the main antibody producing immune cells, to autoimmunity, and particularly of the long-lived plasma cell subset. Such cells can survive for several months (if not years) producing (auto-) antibodies and are resistant to most of the immunomodulatory therapies used in MG, thus representing promising therapeutic targets. Moreover, the *in vitro*, *in vivo* and clinical experience with bortezomib in antibody-mediated autoimmune diseases and organ transplantation is carefully reviewed, with emphasis on the effects on the plasma cell population. Taking this information into account, I proposed and discussed the rationale and relative advantages of bortezomib's

treatment in certain subsets of MG patients, mainly severely-affected or refractory ones. Such patients could indeed benefit from a rapid reduction in autoantibody titers, though bortezomib's side-effects must be carefully taken into consideration when evaluating plasma cell depletion as an autoantibody-reducing therapy.

Chapter 5 demonstrates that plasma cell depletion with bortezomib reduces anti-AChR and IgG levels in the chronic EAMG model, ameliorating the animal's clinical condition concomitantly. The proteasome inhibitor bortezomib has been previously shown to eliminate plasma cells and improve the clinical condition in a mice model of lupus erythematosus. To investigate such effects in MG, bortezomib was tested in a rat EAMG model for an experimental period of eight weeks. One group of rats received bortezomib twice a week subcutaneously right after immunization (8w-Bz), and another group received it 4 weeks after immunization (4w-Bz), while the control group received saline injections. Bortezomib induced apoptosis in bone marrow plasma cells and reduced the amount of plasma cells in the bone marrow by up to 81%. In the EAMG animals, bortezomib efficiently reduced the rise of anti-AChR autoantibody titers and IgG levels. In the 8w-Bz animals, it prevented ultrastructural damage of the postsynaptic membrane, improved neuromuscular transmission, and decreased myasthenic symptoms. These outcomes, however, were not significantly improved in 4w-Bz animals despite their significant autoantibody reduction, which was comparable to that of 8w-Bz animals. This was probably due to transient side-effects of bortezomib and the relatively short-course of treatment, which did not allow for a complete recovery of the NMJ.

Chapter 6 evaluates the effects of proteasome inhibition with bortezomib in primary cultures of human thymic cells from EOMG patients. Frequently, the thymus of EOMG patients is enriched in plasma cells that, after mechanical and enzymatic disruption of the tissue, can produce anti-AChR antibodies in culture for several weeks. The duration of auto-antibody production, its radio-resistance, and its independence from mitogenic stimulation strongly implies plasma cells, primarily the long-lived ones, as the main autoantibody producing cells in these cultures. In this study, it was demonstrated that bortezomib, but not corticosteroids or lenalidomide, killed long-lived plasma cells in cultured thymus cells from EOMG patients and halted their spontaneous production not

only of autoantibodies but also of total IgG. Bortezomib-treated plasma cells showed ultrastructural changes characteristic of endoplasmic reticulum stress after 8 h, and were no longer detectable at 24 h. Consequently, in this chapter it was corroborated that bortezomib is toxic for human autoimmune plasma cells and that its administration, even at 60 times lower doses than the peak values measured in multiple myeloma patients, can lead to a rapid and sustained reduction of autoantibody production.

In conclusion, this thesis explored novel strategies for treating MG and demonstrated that there is still room for improvement in the management of this autoimmune disease, particularly for treatment-refractory patients and those with high autoantibody titers. In this regard, I have shown that long-lived plasma cells are promising therapeutic targets in MG and depleting them is likely to be fundamental for rapidly and significantly reducing autoantibody titers in MG, and possibly other antibody-mediated diseases. Additionally, I investigated the response of the muscle to the autoimmune challenge in MG and provided supporting evidence on how NMJ proteins (such as Dok-7) can influence the susceptibility to MG, which could have potential therapeutic applications in the future. Thus, these findings provide a stepping stone for developing a more comprehensive treatment strategy for MG, both by expanding the therapeutics options for refractory patients and by focusing on muscle-specific aspects of the disease.

Samenvatting

Het doel van dit proefschrift was nieuwe strategieën te bestuderen voor de behandeling van myasthenia gravis (MG), een goed gekarakteriseerde auto-immuunziekte van de neuromusculaire overgang (*neuromuscular junction*, NMJ) die leidt tot spierzwakte en vermoeibaarheid van skeletspieren; MG wordt gewoonlijk behandeld met een combinatie van breed-spectrum immunosuppressiva. In mijn onderzoek heb ik mij geconcentreerd op twee factoren die gewoonlijk niet beschouwd worden als therapeutische targets en die direct betrokken zijn bij de ontwikkeling van de symptomen: de plasmacellen (die het merendeel van de antistoffen produceren) en de reactie van de spier zelf op de auto-immuunaanval.

In **Hoofdstuk 1** wordt de structuur en het functioneren van de NMJ verklaard, waarbij de nadruk wordt gelegd op de karakteristieke eigenschappen van elektrofysiologie en structuur, en de daarmee corresponderende pathologische veranderingen die geassocieerd zijn met MG. In het bijzonder wordt aandacht besteed aan de pathologische mechanismen van de verschillende sub-klassen van auto-antistoffen in de hoofdgroepen van MG: AChR-MG (acetylcholinereceptor-MG; hoofdzakelijk pro-inflammatoire sub-typen IgG1 en IgG3) en MuSK-MG (hoofdzakelijk anti-inflammatoir IgG4). Van deze IgG sub-typen wordt het vermogen om complement te binden en te activeren uitgelegd, alsmede het vermogen van de Fab-arm uitwisseling, de toename van antigene modulatie van AChRs of een functionele blokkade. Daarbij wordt het verlies van meerdere AChR-geassocieerde eiwitten besproken die van belang zijn voor de synaptogenese van de NMJ in MG en CMS *(congenital myasthenic syndromes)* (utrophin, rapsyne, MuSK, Dok-7, Lrp4, Tid1) en het principe van het verlies van AChR-geassocieerde eiwitten als een mogelijke factor die de ernst van MG kan beïnvloeden. Ten slotte worden de voornaamste modellen besproken van MG en de relatieve bijdragen van de verschillende IgG sub-typen op deze modellen.

Hoofdstuk 2 behandelt de reactie van de spier op een auto-immuun aanval die wordt gemedieerd door anti-AChR antistoffen in het chronische EAMG-model *(experimental autoimmune myasthenia gravis)*. Een twee-dimensionaal *difference in-gel electrophoresis* (2D-DIGE) techniek werd toegepast om het proteomisch profiel in verschillende stadia van EAMG te bestuderen. In totaal werden 22 eiwitten gevonden die

differentieel tot expressie werden gebracht, hoofdzakelijk gerelateerd aan metabole en *stress-response pathways*. NMJ eiwitten die differentieel tot expressie kwamen werden niet gevonden, hetgeen waarschijnlijk het gevolg was van het feit dat deze eiwitten een zeer klein onderdeel uitmaken van het arsenaal aan eiwitten in het proteoom van de hele spier en ook vanwege de technische beperking dat de 2D-DIGE techniek niet in staat is membraan-gebonden eiwitten te detecteren. Interessant was dat de differentieel tot expressie gebrachte eiwitten suggereren dat er een switch was opgetreden van het spiervezel type naar de meer vemoeidheid-resistente *slow-twitch* vezels, of een preferentieel verlies van de gemakkelijk vermoeibare *fast-twitch* vezels. Dit fenotype is eerder geassocieerd met andere vormen van spierpathologie met een verslechterde contractiekracht (bijv. *inclusion body myositis*), hetgeen suggereert dat de spier in beide omstandigheden een soortgelijke reactie geeft, nl. switch van *fast* naar *slow-twitch* vezels.

In **hoofdstuk 3** wordt in een passief transfer EAMG-model de rol bestudeerd van Dok-7, een eiwit dat essentieel is voor de embryonale ontwikkeling van NMJs en de volledige activering van MuSK. Aangezien Dok-7 het nieuwste lid is van de familie van eiwitten die betrokken zijn bij de clustering van AChRs, en ook aangezien mutatie in Dok-7 één van de belangrijkste oorzaken is van CMS, werd de hypothese geformuleerd dat een gereduceerde hoeveelheid van Dok-7 in de NMJ de drempel om EAMG te ontwikkelen verlaagt en het herstel remt na een aanval van antistoffen. Om deze hypothese te testen werden anti-Dok-7 shRNA's getransfecteerd in de tibialis anterior spier van volwassen ratten middels electroporatie en werd EAMG 35 dagen later opgewekt. Het silencen van Dok-7 veroorzaakte een significante verslechtering van de neuromusculaire transmissie en bracht een vermindering teweeg van de hoeveelheid AChRs in de NMJ van EAMG dieren die behandeld waren geweest met een subklinische dosis van mAb35 (5 pmol/100 g lichaamsgewicht). In tegenstelling hiermee had de silencing van Dok-7 geen effect op de controle dieren of op het herstel van de auto-immuun aanval tot 14 dagen na het opwekken van EAMG. Echter op dit tijdstip was de NMJ onverwachts nog niet volledig functioneel hersteld. Verder werden geen evidente abnormaliteiten in de morfologie van de NMJs waargenomen met de electronenmiscroscoop na silencing van Dok-7. Dit suggereert dat een gereduceerd niveau van Dok-7 niet kritisch van invloed is voor het

herstel van de NMJ na schade maar wel dat een reductie ervan een rol zou kunnen spelen in de drempel om EAMG te kunnen opwekken, waarschijnlijk door de clusters van AChRs minder resistent te maken tegen aanval met antistof.

In **hoofdstuk 4** worden de mogelijke therapeutische toepassingen besproken van de depletie van plasmacellen middels applicatie van de proteasoomremmer bortezomib in MG. Dit hoofdstuk gaat in op de bijdrage van plasmacellen, die van alle immuuncellen de meeste antistof produceren, aan het fenomeen auto-immuniteit en in het bijzonder de subset van lang-levende plasmacellen. Dit type cellen kunnen gedurende maanden (zo niet jaren) overleven terwijl ze antistoffen produceren die resistent blijken tegen de meeste immunosuppressie-therapieën in MG. Lang-levende plasmacellen zijn derhalve veelbelovende doelwitten voor therapie. Verder worden de *in vivo* en *in vitro* data en de klinische ervaring met bortezomib in antistof-gemedieerde auto-immuunziekten en orgaantransplantatie onderworpen aan een zorgvuldige, genuanceerde beschouwing, met nadruk op de effecten op de populatie van plasmacellen. Deze informatie in beschouwing nemende, heb ik op rationele gronden voorgesteld dat behandeling met bortezomib relatieve voordelen zou kunnen bieden in subsets van MG patiënten, namelijk die hetzij ernstig zijn aangedaan hetzij refractair zijn. Dit type patiënten zouden inderdaad baat kunnen hebben bij vermindering van antistoftiters, alhoewel benadrukt moet worden dat rekening gehouden moet worden met de bijwerkingen van bortezomib wanneer wordt overwogen om door middel van bortezomib het niveau van auto-antistoffen te doen dalen via reductie van plasmacellen.

In **hoofdstuk 5** wordt aangetoond dat depletie van plasma cellen onder invloed van bortezomib de niveau's van anti-AChR antistoffen en IgG verlagen in het chronische EAMG- model, waardoor de klinische conditie van het dier dienovereenkomstig wordt verbeterd. Eerder was aangetoond dat de proteasoomremmer bortezomib tot eliminatie leidt van plasmacellen en ook de klinische conditie verbetert van muizen in een model voor lupus erythematosus. Om dergelijke effecten in MG te onderzoeken, werd bortezomib getest in een rat EAMG-model gedurende een periode van acht weken. Eén groep ratten kreeg tweemaal per week subcutaan bortezomib onmiddellijk na immunisatie (8w-Bz) en een tweede groep kreeg het geneesmiddel 4 weken na

immunisatie (4w-Bz); de controlegroep kreeg injecties met fysiologisch zout. Bortezomib veroorzaakte apoptosis in plasmacellen van het beenmerg en veroorzaakte een vermindering in het aantal plasmacellen in het beenmerg tot 81%. In EAMG dieren leidde toediening van bortzeomib tot een vermindering van de toename van anti-AChR antistoftiters en niveau van het IgG. In de groep van 8w-Bz dieren verhinderde het de ultrastructurele schade van het postsynaptisch membraan, verbeterde het de neuromusculaire transmissie en verminderde het de myasthene symptomen. Deze effecten werden echter niet significant verbeterd in de groep van 4w-Bz dieren ondanks het feit dat er een significante daling was in de titer van auto-antistof die van dezelfde grootte was als die in de 8w-Bz dieren. Waarschijnlijk was dit het gevolg van voorbijgaande neveneffecten van bortezomib en de relatieve korte duur van de behandeling die niet toereikend was voor een volledig herstel van de NMJ.

In **hoofdstuk 6** worden de effecten geëvalueerd van proteasoomremming met bortezomib in primaire culturen van humane thymuscellen afkomstig van EOMG-patiënten *(early-onset MG)*. In veel gevallen is de thymus van EOMG-patiënten verrijkt in plasmacellen die, na mechanische en enzymatische disruptie van het weefsel, het vermogen hebben tot productie van anti-AChR antistoffen gedurende enkele weken in weefselkweek. De duur van auto-antistofproductie, de ongevoeligheid voor bestraling en de weerstand tegen mitogene stimulatie geven een sterke aanwijzing dat plasmacellen, en in het bijzonder het lang-levende type, de voornaamste bron is van auto-antistoffen in deze kweken. In dit onderzoek hebben we aangetoond dat bortezomib, maar niet corticosteroïden of lenalidomide, lethaal was voor lang-levende plasmacellen in thymus-celkweken van EOMG-patiënten en de productie stopzette van auto-antistoffen alsmede die van het totale IgG. Plasmacellen die waren behandeld met bortezomib vertoonden ultrastucturele veranderingen na 8 uur die karakteristiek zijn voor stress van het endoplasmatisch reticulum; cellen waren niet langer detecteerbaar na 24 uur. Dientengevolge is in dit hoofdstuk aannemelijk gemaakt dat bortezomib toxisch is voor humane auto-immuun plasmacellen en dat toediening ervan, zelfs in een concentratie die 60 maal lager is dan de piek concentratie die gevonden was in multipele myeloma patiënten na bortezomib behandeling, kan leiden tot een blijvende vermindering van de

productie van auto-antistoffen.

Samenvattend is in dit proefschrift een verkenning uitgevoerd voor nieuwe strategieën om MG te behandelen. We hebben hierbij aangetoond dat er nog ruimte voor verbetering is voor de behandeling van deze auto-immuunziekte, in het bijzonder voor de behandeling van patiënten die ongevoelig blijken voor behandeling alsmede patiënten met hoge titers van auto-antistoffen. Wat dit betreft heb ik laten zien dat lang-levende plasmacellen belofte inhouden als therapeutisch target in MG. Het is essentieel om juist deze cellen op te ruimen teneinde het niveau van auto-antistoffen in MG te kunnen reduceren. Deze aanpak zou tevens kunnen werken in andere auto-immuun aandoeningen. Verder heb ik onderzocht wat de response van de spier was op een auto-immuun challenge in MG en daarmee aanvullend bewijs gevonden hoe verlaging van NMJ eiwitten (zoals Dok-7) de vatbaarheid voor MG kan beïnvloeden, hetgeen therapeutisch potentieel voorspelt voor de toekomst. Derhalve, leveren deze data een *stepping stone* voor de ontwikkeling van een uitgebreide behandelingsstrategie in MG zowel door de therapeutische opties uit te breiden in refractoire patiënten als door te focusseren op spier-specifieke aspecten van deze ziekte.

Resumen

Esta tesis tiene como objetivo principal el desarrollo de nuevas estrategias para un tratamiento mas exhaustivo de la enfermedad autoinmune miastenia gravis (MG). MG es una enfermedad autoinmune prototípica que afecta la unión neuromuscular (UNM), causando debilidad muscular y fatigabilidad, y que es tradicionalmente tratada con una combinación de agentes inmunomoduladores de amplia gama. Para cumplir los objetivos de esta tesis, me he enfocado en dos factores que usualmente no se tienen en cuenta como posibles opciones de tratamiento, y que están directamente relacionados en el desarrollo de los síntomas en MG: las células plasmáticas (principales productoras de autoanticuerpos) y la respuesta del músculo ante el ataque de los autoanticuerpos.

El **Capitulo 1** explica la estructura y el funcionamiento de la UNM, haciendo hincapié en sus principales características electrofisiológicas y estructurales, y las correspondientes alteraciones patológicas de la misma asociadas con MG. En particular, se explican los mecanismos patológicos de las diferentes subclases de autoanticuerpos en los principales subtipos de MG: MG con anticuerpos hacia el receptor de acetilcolina (AChR-MG, predominantemente subclases pro-inflamatorias IgG1 y IgG3) y con autoanticuerpos hacia la quinasa-especifica del músculo (MuSK-MG, predominantemente de la subclase anti-inflamatoria IgG4). Para cada una de estas subclases de inmunoglobulinas se describen exhaustivamente sus capacidades para interaccionar con el sistema del complemento y activarlo, realizar intercambio de brazos Fab, incrementar la modulación antigénica de AChRs, o bloquear funcionalmente el AChR. Además, se elabora sobre la perdida de proteínas asociadas al AChR que son importantes para la sinaptogénesis neuromuscular (utrophin, rapsyn, MuSK, Dok-7, Lrp4, Tid1) tanto en MG como en los síndromes miasténicos congénitos (SMC), y se introduce el concepto de la perdida de proteínas asociadas al AChR como posible factor contribuyente a la severidad de los síntomas en MG. Finalmente, se describen los principales modelos animales de MG, y la contribución relativa de las diferentes subclases de inmunoglobulinas a los cambios patológicos en cada modelo.

El **Capitulo 2** estudia la respuesta del músculo ante un ataque autoinmune mediado por anticuerpos contra el AChR (anti-AChR) en el modelo crónico de EAMG *(experimental autoimmune myasthenia gravis,* por sus siglas en inglés*)*. A fines de analizar

el perfil proteómico del músculo en diferentes estadios clínicos de EAMG, se utilizo la técnica de electroforesis bidimensional diferencial (2D-DIGE, por sus siglas en inglés). En total se identificaron 22 proteínas expresadas diferencialmente en el músculo, en su mayoría relacionadas con vías metabólicas y de respuesta al estrés celular. Proteínas de la UNM no fueron identificadas entre las proteínas expresadas diferencialmente en músculos de animales con EAMG, probablemente debido a la relativamente baja abundancia de estas proteínas en el proteoma muscular, y también debido a limitaciones técnicas de la 2D-DIGE para detectar proteínas unidas a membranas lipídicas. Es de particular interés el hecho de que estas modificaciones en la expresión de proteínas sugieren un cambio en el tipo de fibras musculares hacia tipos de fibras más resistentes a la fatiga (las fibras de contracción lenta), o bien reflejan una pérdida preferencial de las fibras de contracción rápida (las cuales se fatigan más rápidamente). Este tipo de cambio fenotípico ha sido anteriormente asociado con otras patologías que afectan la contracción muscular (ej. miositis de cuerpos de inclusión), lo que sugiere una respuesta similar del músculo ante este tipo de patologías.

El **Capitulo 3** investiga el rol de la proteína adaptadora de la UNM Dok-7, la cual es crucial para el desarrollo embrionario de UNMs y la activación completa de MuSK, en el modelo de transferencia pasiva de EAMG. Teniendo en cuenta que Dok-7 es la proteína de las vías de *clustering* del AChR más recientemente identificada, y que mutaciones en Dok-7 son una de las principales causas de SMC, se hipotetizó que una reducción en los niveles de Dok-7 en la UNM aumenta la susceptibilidad para desarrollar EAMG y afecta la recuperación de la UNM luego del ataque de los autoanticuerpos. Para corroborar esta hipótesis, shARNs (*short hairpin* ARN, por sus siglas en inglés) específicos para Dok-7 fueron transfectados en el músculo *tibialis anterior* de ratas adultas mediante electroporación *in vivo*, y el modelo de EAMG fue inducido 35 días mas tarde. La silenciación de Dok-7 empeoró la transmisión neuromuscular y redujo los niveles de AChR en la UNM en animales con EAMG que recibieron una dosis subclínica de mAb35 (*monoclonal antibody 35*, por sus siglas en inglés) (5 pmol/100g peso). En contraste, la silenciación de Dok-7 no tuvo efectos significativos tanto en los animales control como en la recuperación de la UNM del ataque de autoanticuerpos en animales con EAMG (14

pmol/100g peso), hasta 14 días luego de la inducción del modelo. En este estadio del experimento, no obstante, la UNM aún no estaba completamente recuperada en su funcionalidad, lo cual fue inesperado. Adicionalmente, no se observaron claras alteraciones morfológicas por microscopia electrónica en UNMs en las cuales la expresión de Dok-7 fue silenciada. Estos resultados sugieren que una reducción en la expresión de Dok-7 no es esencial para la recuperación de la UNM una vez que ésta esta dañada, aunque sí podría desempeñar un papel importante en la susceptibilidad para desarrollar EAMG, probablemente a causa de una reducción en la resistencia de los *clusters* de AChR hacia el ataque de autoanticuerpos.

El **Capitulo 4** discute las posibles aplicaciones terapéuticas de la depleción de células plasmáticas con el inhibidor del proteasoma bortezomib en el tratamiento de MG. En este capitulo se elabora sobre la contribución relativa de las células plasmáticas, las principales células productoras de anticuerpos del sistema inmune, en enfermedades autoinmunes y, en particular, sobre la importancia del subgrupo de células plasmáticas de larga vida en este tipo de enfermedades. Este subgrupo es capaz de sobrevivir por muchos meses (y quizás años) produciendo (auto-) anticuerpos y son resistentes a la mayoría de las terapias inmunomoduladoras que se utilizan en MG, representando, por lo tanto, nuevas y prometedoras dianas terapéuticas. Además, se provee una revisión bibliográfica meticulosa de la experiencia con bortezomib *in vitro*, *in vivo* y en pacientes con enfermedades autoinmunes mediadas por anticuerpos o con transplante de órganos, enfatizando los efectos de esta droga sobre la población de células plasmáticas. Teniendo en cuenta esta información, yo propongo los principios, y discuto las ventajas relativas, del uso de bortezomib en el tratamiento de ciertos subgrupos de pacientes con MG, principalmente aquellos con síntomas severos o refractarios al tratamiento estándar. Estos subgrupos de pacientes podrían beneficiarse enormemente de una reducción rápida en los niveles de autoanticuerpos, aunque los efectos adversos de bortezomib deben tenerse cuidadosamente en consideración cuando se evalúa la reducción de células plasmáticas con este medicamento como una posible terapia para reducir autoanticuerpos.

El **Capitulo 5** demuestra que la depleción de células plasmáticas con bortezomib

reduce los niveles de anticuerpos anti-AChR y de IgG en el modelo crónico de EAMG, concomitantemente mejorando el estado clínico de los animales. Previamente, se había demostrado que el inhibidor del proteasoma bortezomib elimina células plasmáticas y mejora la condición clínica en un modelo murino de lupus eritematoso sistémico. A fines de investigar éstos efectos en MG, bortezomib fue testado en un modelo de EAMG en ratas durante un periodo de ocho semanas. Un grupo de ratas recibió bortezomib dos veces por semana de forma subcutánea inmediatamente después de la inmunización (8w-Bz, por sus siglas en inglés), y otro grupo lo recibió cuatro semanas después de la inmunización (4w-Bz), en tanto que el grupo control recibió inyecciones de solución salina fisiológica. Bortezomib indujo apoptosis en las células plasmáticas de la médula ósea y redujo la cantidad de células plasmáticas en este tejido en hasta un 81%. En los animales con EAMG, bortezomib redujo eficientemente el incremento en los niveles de autoanticuerpos anti-AChR y de IgG. En los animales del grupo 8w-Bz, previno el daño estructural de la membrana post-sináptica, mejoró la transmisión neuromuscular, y redujo los síntomas miasténicos. Sin embargo, éstos parámetros no fueron significativamente mejorados en los animales del grupo 4w-Bz, a pesar de la significativa reducción en sus niveles de autoanticuerpos, que era comparable con la reducción observada en animales del grupo 8w-Bz. Esto fue probablemente debido a efectos secundarios transitorios de bortezomib y al relativamente breve período de tratamiento con esta droga, el cual no permitió una recuperación completa de la UNM.

El **Capitulo 6** evalúa los efectos de la inhibición del proteasoma con bortezomib en cultivos primarios de células tímicas de pacientes con MG de aparición temprana (EOMG, *early-onset* MG, por sus siglas en inglés). Frecuentemente, el timo de pacientes con EOMG contiene células plasmáticas que, luego de la disrupción mecánica y enzimática del tejido para su cultivo, pueden producir anticuerpos anti-AChR *in vitro* por varias semanas. La prolongada producción de autoanticuerpos, la radio-resistencia de la misma, y su independencia de la estimulación mitogénica, implican vehementemente a las células plasmáticas, principalmente las de larga vida, como las mayores productoras de autoanticuerpos en estos cultivos celulares. En este estudio se demostró que bortezomib, pero no los corticoides o lenalidomide, elimina las células plasmáticas de larga vida en

cultivos de células tímicas de pacientes con EOMG y detiene la producción espontánea, no solo de autoanticuerpos, sino que también de IgGs. Las células plasmáticas tratadas con bortezomib presentaron cambios ultra-estructurales característicos de estrés en el retículo endoplasmático y apoptosis luego de 8 horas en presencia de bortezomib y, luego de 24 horas, ya no pudieron ser detectadas. Consecuentemente, en este capitulo se corroboró que bortezomib es tóxico para células plasmáticas autoinmunes de origen humano y que su administración, aún en dosis 60 veces menores a los valores máximos observados en pacientes con mieloma múltiple, podría conducir a una rápida y sostenida reducción en la producción de autoanticuerpos.

Para concluir, esta tesis exploró nuevas estrategias para tratar la MG y demostró que aún existen oportunidades para mejorar el manejo de esta enfermedad autoinmune, particularmente en el tratamiento de pacientes refractarios y aquellos con altos niveles de autoanticuerpos. En este sentido, yo he demostrado que las células plasmáticas de larga vida son prometedoras dianas terapéuticas en MG y que su depleción probablemente sea fundamental para rápida y significativamente reducir los niveles de autoanticuerpos en MG, y posiblemente también en otras enfermedades mediadas por anticuerpos. Adicionalmente, he investigado la respuesta del músculo ante el ataque autoinmune en MG y he encontrado evidencia sustentable de cómo proteínas de la UNM (e.g. Dok-7) pueden influir la susceptibilidad para el desarrollo de MG, lo cual podría tener potenciales aplicaciones terapéuticas en el futuro. Por consiguiente, estos descubrimientos proveen un punto de partida para el desarrollo de una estrategia de tratamiento más comprensiva para MG, tanto por la expansión de las opciones terapéuticas para pacientes refractarios como por el novedoso enfoque en aspectos músculo-específicos de la enfermedad.

About the author

Curriculum vitae

Alejandro Martin Gomez was born on the 30th May 1983 in Concordia, Argentina. In 2000 he obtained his High School Degree with specialization in Biological Sciences at the Instituto Nuestra Señora de Los Angeles (Concordia, Argentina), with distinctions for his marks in Biology and Laboratory Work. He was also recognized for his five-year participation at the Science Club of the abovementioned institute, an extracurricular activity for conducting and guiding science projects at the high school level. In 2001 he started his higher education studies at the Universidad Nacional del Litoral (UNL, Santa Fe, Argentina) and obtained his degree in Biochemistry in 2007, with the highest grade point average of his class. In 2005, he was awarded with a scholarship from the Santander Central Hispano Undergraduate Exchange Program to study one semester of the Biochemistry Specialization at the Universidad Autónoma de Madrid (Madrid, Spain). Between 2006 and 2007, he conducted his final research internship at the laboratory of Metabolic and Nutrition Related Diseases (UNL, Santa Fe, Argentina), under the supervision of Prof. Yolanda B. de Lombardo. His final project was entitled ¨Effects of Soya Protein in Rats with Lipids Disorder and Glucose Intolerance¨, which explored the effects of a soya protein-based diet in a rat model of metabolic syndrome. In 2007, he worked as medical technologist at the Clinical Biochemistry Laboratory of Rawson Clinic and the Jose Maria Cullen Hospital in Santa Fe, Argentina. In 2008, he obtained a Marie-Curie Fellowship that allowed him to start his PhD at the division Neuroscience in Maastricht University (The Netherlands), under the supervision of Prof. Dr. Marc H. De Baets, Mario Losen and Pilar Martinez-Martinez. In his PhD project, he investigated pathological mechanisms and potential new therapies for the neuromuscular autoimmune disease myasthenia gravis. The results of his work are described on this thesis.

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** These authors contributed equally to this work*

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- 3rd Marie Curie Fellow Meeting. March 25th, 2009, Maastricht (The Netherlands)
- 3rd Aachen-Maastricht Neuromuscular Research Meeting. October 2009, Maastricht (The Netherlands)
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- 16th Euron PhD Days. September 27th-28th, 2012, Maastricht (The Netherlands)
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- Final Marie Curie Fellow Meeting. March 16th, 2010, Maastricht (The Netherlands)
- 3rd MHeNS Research Day. March 17th, 2010, Maastricht (The Netherlands)
- 7th International Congress on Autoimmunity. May 5th-9th, 2010, Ljubljana (Slovenia)
- 10th International Congress of Neuroimmunology. October 26th-30th, 2010, Sitges (Spain)

*** Gomez, A.M.**, Stevens, J., Molenaar, P., Losen, M., De Baets, M.H., Martinez-Martinez, P. *Repair mechanisms at the neuromuscular junction – the role of Dok-7.*

- 15th Euron PhD Days. September 22nd-23rd, 2011, Bad Honnef (Germany)
- 12th International Conference on Myasthenia Gravis and Related Disorders. 21st-23rd May, 2012, New York (USA)
- 6th MHeNS Research Day. June 6th, 2013, Maastricht (The Netherlands)
- Myasthenia 2013. 1st-2nd July, 2013, Paris (France)

* Gomez, A.M., Vanheel, A., Losen, M., Molenaar, P.C., De Baets, M.H., Noben, J.P., Hellings, N., Martinez-Martinez, P. *Proteomic analysis of rat tibialis anterior muscles at different stages of experimental autoimmune myasthenia gravis*
- Myasthenia 2013. 1st-2nd July, 2013, Paris (France)

Travel awards

- Young Investigator Travel Fellowship. *(Myasthenia Gravis Foundation of America)*, 12th International Conference on Myasthenia Gravis and Related Disorders, May 2012, New York (NY) (1250 USD).
- Trainee Abstract Award. 100th Annual Meeting of the American Association of Immunologists (AAI) - Immunology 2013. May 2013, Honolulu (HI) (750 USD).
- Travel Fellowship. Myasthenia 2013. July 2013, Paris (France) (300 EUR)

Courses & Internships

- Euron Course Neuroimmunology. May 2008, Hasselt University, Belgium.
- Course on Laboratory Animal Science. September 2008, Division of Laboratory Animal Science, Utrecht University.
- Dutch Course, Module 1 (NL-9A1). September – December 2008, Language Centre, Maastricht University.
- Course on Radiological Protection, Level 5b. February - March 2009, Department of Radiological Laboratories, Maastricht University.
- Dutch Course, Module 2 (NL-9A2). April – June 2009, Language Centre, Maastricht University.
- Basic Course for Tutors in Problem-Based Learning. June 2009, Institute for Education (FHML), Maastricht University.
- Academic Visit to David Beeson's Lab at the Department of Clinical Neurology, University of Oxford. February 2010, Oxford, UK.
- Dutch Course, Module 3 (NL-9B1-). February – May 2010, Language Centre, Maastricht

University.

- 10th Course of the European School of Neuroimmunology (ESNI). October 26th, 2010, Sitges, Spain.

- Course in Academic Writing for PhD's (PhD-1). October - November 2010, Language Centre, Maastricht University.

- PhD Presentations Course (PhD-4). March – May 2012, Language Centre, Maastricht University.

- Course in Academic Writing for Biomedical Sciences (PhD-2). September - October 2012, Language Centre, Maastricht University.

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